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REQUIREMENTS OF PRECURSORS FOR THE SYNTHESIS OF VITAMIN B_{12} IN CEREAL MATRICES

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ABSTRACT

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Tiivistelmä — Referat — Abstract

To acquire sufficient amount of vitamin B_{12} (2-3 µg/day), humans mostly consume animal-derived foods in their diet, making vegetarians and vegans highly susceptible to vitamin B_{12} deficiency. Although sufficient vitamin B_{12} intake can also be met by consuming fortified foods and vitamin B_{12} supplements, there is an increasing trend to consume natural and food grade products rather than supplements in tablet form. However, plant-based fermented foods that contain naturally synthesized vitamin B_{12} are limited. This study therefore aimed to investigate the requirement of different precursors in the biosynthesis of vitamin B_{12} in optimal media conditions and then in cereal matrices using strains of *Propionibacterium freudenreichii*.

Vitamin B_{12} was produced by three selected *P. freudenreichii* strains (strain 1, 2 and 3) in supplemented whey permeate (SWP) medium, 30% w/v barley malt matrix and 6% barley flour matrix. The additions of 5 mg/L cobalt chloride, 15 mg/L DMBI, 15.05 mg/L riboflavin and 3.29 g/L nicotinamide were investigated. The effects of three different time points (0, 72, 144 h) in adding riboflavin and nicotinamide were also investigated. Depending on the type of precursors and the timing of adding the precursors, six conditions were tested in the SWP medium, while three conditions were tested in the cereal matrices. After extraction in the presence of sodium cyanide, vitamin B_{12} was then quantified by microbiological assay (MBA) and ultra high-performance liquid chromatography (UHPLC).

The two-stage fermentation of 72-hour anaerobic/96-hour aerobic fermentation led to high vitamin B_{12} yields with maximum production of 2.20 µg/mL and 1.41 µg/g produced in the SWP medium and the barley malt matrix, respectively. Depending on the type of precursors added in the culture samples, the capacity of the *P. freudenreichii* strains in vitamin B_{12} production varied. The strain 3 produced maximum vitamin B_{12} yields with the addition of riboflavin and nicotinamide at 0 h. With the vitamin B_{12} amount achieved in the fermented cereal matrices, this study successfully demonstrated the promising possibilities to enrich plant-based foods with vitamin B_{12} through *in situ* fermentation.

Avainsanat — Nyckelord — Keywords

Vitamin B_{12}, *Propionibacterium freudenreichii*, cereal matrix, riboflavin, nicotinamide.

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PREFACE

This study was carried out in collaboration with the research groups from the division of Food Chemistry, Cereal Technology and Dairy Technology, at the Department of Food and Environmental Sciences, Faculty of Agriculture and Forestry, University of Helsinki. The study was a part of the on-going doctoral research on vitamin B_{12} conducted by one of my supervisors, Bhawani Chamlagain.

Firstly, I would like to express my sincere gratitude to Professor Vieno Piironen for offering the opportunity to be involved in this sophisticated project. I thank her very much for organizing the working possibility and for providing thesis grant during this research. My deep gratitude goes to Bhawani Chamlagain for being an exceptional tutor throughout the work. Without his guidance, advice, direct supervision and tremendous patience, the completion of this work would not have been possible.

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At last, I would like to express my greatest gratitude to my superb family, Theng Agus Hartanto (the awesome Dad), Liliani Setio Kartono (I applaud you for being a tiger Mom), Iswati Setio Kartono (my greatest aunt and personal physician) and Harun Reza Sugito (I'm so proud of you Bro!!) for their unconditional love, encouragements, motivations and prayers in every step of my life. To my boyfriend, Ricky Lim, I really thank you for your sacrifice and always patiently listening to my complaints and weariness. Above all, I would like to praise and thank my saviour, Jesus Christ for the abundant blessings undeservingly given to me.

Helsinki, Finland

Tessa Ayuningtyas Sugito

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
AMN	Amnionless
AdoCbl	Adenosylcobalamin
ALA	5-aminolevulenic acid
ALAS	5-aminolevulinic acid synthase
BMM	Barley malt matrix
BFM	Barley flour matrix
CNCbl	Cyanocobalamin
CUBN	Cubilin
DMBI	5,6-dimethylbenzimidazole
FMN	Flavin mononucleotide
GluTR	Glutamyl-tRNA reductase
GRAS	Generally recognized as safe
GSA	Glutamate-1-semialdehyde
GSAM	Glutamate-1-semialdehyde-2, 1-aminomutase
GSH	Glutathione
HSS	High Strength Silica
LAB	Lactic acid bacteria
LLD	*Lactobacillus lactis* Dorner
LMBD1	Cobalamin F protein
LOQ	Limit of quantitation
MBA	Microbiological assay
MeCbl	Methylcobalamin
mL	Millilitre
MMA	Methylmalonic acid
MMAA	Cobalamin A protein
MMAB	Cobalamin B protein
MMACHC	Cobalamin C protein
MMADHC	Cobalamin D protein
MRP1	Multidrug resistance protein 1
MTRR	Cobalamin E protein
MUT	(R)-methyl-malonyl-CoA mutase
NADPH	Glutamate synthase
ng	Nanogram
OD	Optical density
PBGD	Porphobilinogen deaminase
PBGS	Porphobilinogen synthase
PCR	Polymerase chain reaction
PLP	Pyridoxal 5’-phosphate
QPS	Qualified presumption of safety
RDA	Recommended daily allowance
SAM	S-adenosyl-L-methionine
SEM	The standard error of the mean
SUMT	S-adenosyl-L-methionine uroporphyrinogen III methyltransferase
SSF	Solid substrate fermentation
SWP	Supplemented whey permeate
THF	Tetrahydrofolate
UHPLC	Ultra High-Performance Liquid Chromatography
UROS	Uroporphyrinogen III synthase

1 INTRODUCTION

Vitamin B_{12} inarguably has been one of the most complex and fascinating molecules in the world of science and medicine. The vitamin was accidentally discovered in the early 1920s, when it was demonstrated as the cure of pernicious anaemia. As the last vitamin to be isolated, the water-soluble vitamin has the most complex structure and the largest molecular mass (1335-1580 Da) (Martens et al. 2002; Truswell 2007), consisting a planar ring system with cobalt as central atom, a lower (alpha) ligand and an upper (beta) ligand. Over the span of more than 90 years, much has been revealed about the vitamin that was previously described as 'frighteningly complex'. Yet, many missing pieces in the whole vitamin B_{12} saga are still waiting to be found, particularly vitamin B_{12} production in plant-based food system.

In the metabolism of mammalian cells, vitamin B_{12} is required to assist two enzymatic activities; methionine synthase and (R)-methyl-malonyl-CoA mutase. Methionine synthase is involved in the DNA synthesis. While (R)-methyl-malonyl-CoA mutase requires vitamin B_{12} in the catabolism of odd-chain fatty acids, certain amino acids and cholesterol. When vitamin B_{12} is lacking, the level of homocysteine increases, posing a major risk for heart disease, atherosclerosis, stroke and vascular disease. Uniquely, the biosynthesis of vitamin B_{12} exists only in some bacteria and archaea. Although animals (including humans) and protists require vitamin B_{12}, they do not synthesize it. The vitamin is thus available only in foods fermented or contaminated by such bacteria or in the tissues of animals fed by vitamin B_{12}-containing feed. Hence, humans obtain vitamin B_{12} by mainly consuming animal-derived products.

Due to their diet, vegetarians and vegans are highly prone to vitamin B_{12} deficiency. Since vitamin B_{12} takes part in DNA synthesis, deficiency hinders cell nuclei division that affects blood cell formation, resulting megaloblastic anaemia. In severe vitamin B_{12} deficiency, neuropathy diseases such as dementia and Alzheimer's can also occur. Nevertheless, the trends toward vegetarianism and veganism have been rising due to the increasing awareness of health (Campbell et al. 1998; Daviglus et al. 2008), sustainability issues and animals welfare. To meet the recommended amount (the lowest requirement of any known essential nutrients) at 2.4 µg/d (the Institute of Medicine, 1998), vegetarians and vegans are required to consume fortified foods and/or vitamin B_{12} supplements. However, the recent trends also show that the consumers prefer natural and food grade products to supplements in tablet form. Therefore, more

studies (Hugenholtz and Smid 2002; Hugenholtz 2008; LeBlanc et al. 2011; Capozzi et al. 2012) have been carried out to produce vitamin B_{12} directly in the food system utilizing food-grade microorgansims, called *in situ* vitamin production. Besides providing vitamin B_{12} in foods through natural enrichment, *in situ* fermentation is more cost effective and environment-friendly as chemical processes in the commercial vitamin B_{12} production such as extraction, purification and crystallization can be omitted.

Despite the opportunities to produce vitamin B_{12} through *in situ* fermentation, plant-based fermented foods that contain naturally synthesized vitamin B_{12} are still limited. Previous studies mostly covered the vitamin B_{12} production in tempe/tempeh, an indigenous product of fermented soybean from Indonesia (Liem et al. 1977; Okada 1989; Keuth and Bisping 1993, 1994; Wiesel et al. 1997). In addition, published results on production of vitamin B_{12} by *Propionibacteria* (one of the highest vitamin B_{12}-producing bacteria) in plant-based foods does not exist although it has potential applications in enriching plant-based foods with vitamin B_{12}. Thus, this study aimed to investigate the requirement of different precursors in the vitamin B_{12} biosynthesis in optimal media conditions and then in cereal matrices using strains of *P. freudenreichii*. The study also investigated any differences of vitamin B_{12} production among the strains of *P. freudenreichii* with respect to the added precursors.

To understand the complexity and the importance of vitamin B_{12}, the literature review started with the sophisticated structure of vitamin B_{12} and the availability of vitamin B_{12} in certain foods. The review was followed by how vitamin B_{12} plays important roles in human metabolisms and how a human body transports and absorbs the vitamin. The biosynthesis of vitamin B_{12} and the formation of the lower ligand 5,6-dimethylbenzimidazole (DMBI) were also explained, followed by the coverage of recent studies in optimizing vitamin B_{12} production using plants and waste media. In the next part of the thesis, the methods to prepare the cultures and produce vitamin B_{12} with various treatments were clearly described. The vitamin B_{12} quantification was done by using microbiological assay (MBA) and a newly developed UHPLC method (Chamlagain et al. submitted). The final parts of the thesis presented the results and the discussions on the effects of adding different precursors and varying the timing of adding the precursors on vitamin B_{12} concentrations.

2 LITERATURE REVIEW

2.1 History

The importance of vitamin B_{12} in medical and scientific world was accidentally acknowledged in the early 1920s, when it was demonstrated as the cure of a gastric autoimmune disease, pernicious anaemia. The firstly recorded experiments were performed by an American physician, George Hoyt Whipple, who intentionally bled dogs to induce anaemia. He later fed them with various diets to find the most effective food that brought haemoglobin level back to normal. The result showed that large consumption of pig liver allowed the fastest recovery (Whipple 1927; Robscheit-Robbins and Whipple 1929). Hence, Whipple hypothesized that liver consumption might treat pernicious anaemia (Whipple 1934).

The notable progress in finding the cure of the disease was contributed by the other two American physicians; George Richards Minot and William Parry Murphy (1926). After several clinical studies, they isolated some substances in the liver and discovered iron was the cure of anaemia in dogs. However, they later also found different liver substance, called “extrinsic factor” that cured pernicious anaemia in humans. Albeit this finding, the patients were still treated with high consumption of raw liver or liver juice. In 1934, their discoveries concerning liver therapy in cases of anaemia rewarded Minot and Murphy (together with Whipple) Nobel Prizes in Physiology or Medicine.

For the next 20 years, the identification of the extrinsic factor was still obscure, until some chemists sponsored by pharmaceutical companies isolated a red crystalline compound from liver juice that was used to treat pernicious anaemia. Mary Shaw Shorb (1947, 1948) and Karl August Folkers (Rickes et al. 1948) in collaborative project with Merck developed *Lactobacillus lactis* Dorner (LLD) assay. The LLD assay achieved rapid isolation of the extrinsic factor leading to its purification and characterization. Simultaneously, British researchers, E.L. Smith at Glaxo, and Alexander Robertus Todd performed the same experiment (Smith 1948a, 1948b, 1952). The red crystalline compound was later named vitamin B_{12}.

It took several years later when Dorothy Crowfood Hodgkin and her team specified the three-dimensional structure of vitamin B_{12} (cyanocobalamin) and, 5 years later, coenzyme B_{12} (adenosylcobalamin) based on X-ray crystallographic data (Hodgkin et

al. 1954, 1956, 1957; Lenhert and Hodgkin 1961). Since then, other forms of vitamin B_{12} and pseudo-vitamin B_{12} have been discovered.

2.2 Structure and Forms of Vitamin B_{12}

Vitamin B_{12} was the last vitamin to be isolated. Amongst of vitamins, it has the most complex structure and largest molecular mass (1335-1580 Da) (Martens et al. 2002; Truswell 2007). The water-soluble vitamin covers various polycyclic compounds of the cobalamin group, whose molecules can be characterized into three parts (Figure 1): a planar ring system with cobalt as central atom, a lower (alpha) ligand and an upper (beta) ligand. The planar ring system consists of four pyrrole units (A, B, C, and D, Figure 1) forming cobyrinic acid hexaamide (cobyric acid, factor V_{1a}). The alpha ligand is supplied by the N7-atom of 5,6-dimethylbenzimidazole (DMBI), which is additionally linked to the propionic acid chain of cobyric acid via D-1-amino-propanol-2 and phophoribose, forming nucleotide loop. In some anaerobic bacteria, the alpha ligand is replaced by adenine, forming pseudo-vitamin B_{12} (Martens et al. 2002). The different compounds in vitamin B_{12} structure can be defined as follows (Kumar et al. 2010):

- Cobalamin: the complete vitamin B_{12}
- Corrinoids represent the sum of cobalamin and analogues.
- Vitamin B_{12} analogues include cobyrinic acid hexaamide, cobinamide and cobamide.
- Cobyrinic acid hexaamide (cobyric acid, factor V_{1a}): cobalamin molecule without the nucleotide loop and D-1-amino-propanol-2 linkage.
- Cobinamide: it is the cobyric acid with D-1-amino-propanol-2 linkage.
- Cobamide: cobalamin molecule without the DMBI
- Pseudo-vitamin B_{12}: when DMBI is replaced by adenine.

In biological system, the upper ligand is either an adenosyl group (5'-deoxyadenosylcobalamin) or a methyl group, giving adenosylcobalamin (AdoCbl, coenzyme B_{12}) or methylcobalamin (MeCbl), which supply 10-25% and 75-90%, respectively, of the body pool of circulating cobalamin (Wheatley 2006). The upper ligand can also be substituted by hydroxyl ion, water molecule or cyanide group, forming hydroxycobalamin, aquocobalamin and cyanocobalamin (CNCbl), respectively. MeCbl and AdoCbl are sensitive to light exposure, resulting photochemical degradation to hydroxocobalamin and aquocobalamin at room

temperature in aqueous solution (Kerwar et al. 1971). Hence, pharmaceutical industry produces vitamin B_{12} in the form of CNCbl that is more stable due to the presence of cyanide.

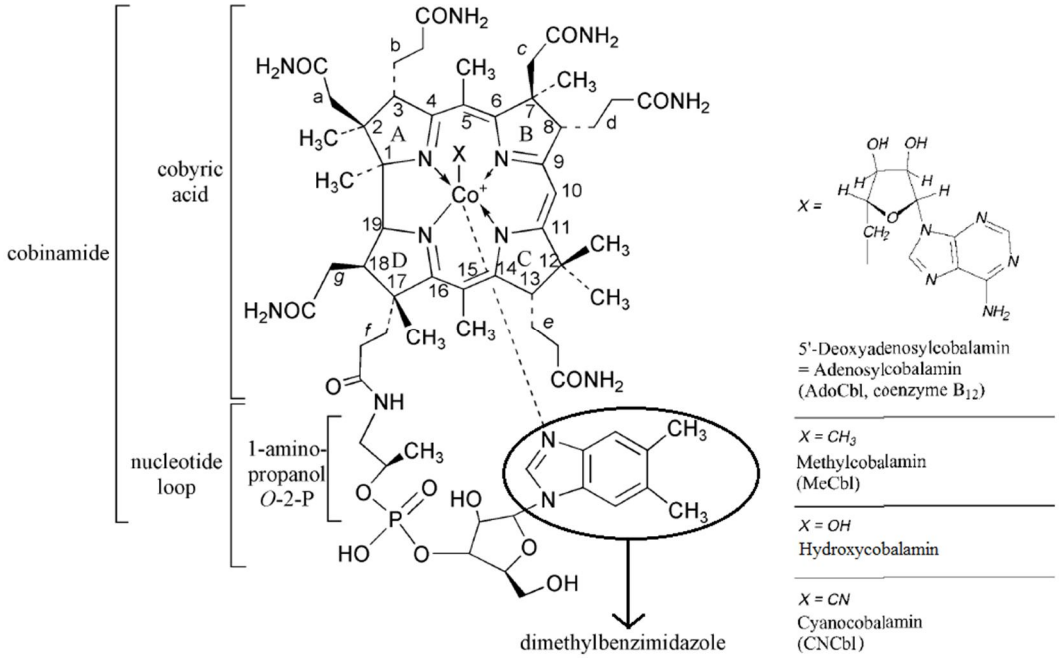

Figure 1. The structure of vitamin B_{12} and some of its derivatives. (Martens et al. 2002; Warren et al. 2002).

2.3 Vitamin B_{12} in Foods

As the synthesis of cobalamin exists solely in some bacteria and archaea, the vitamin is therefore found only in foods fermented or contaminated by such bacteria. Other vitamin B_{12}-containing foods are products derived from the tissues of animals which have been fed by vitamin B_{12}-containing feed or which have obtained it from vitamin B_{12}-producing bacteria of their commensal microflora (Burgess et al. 2009). The vitamin B_{12}-producing bacteria live in the digestive tracts of ruminants and synthesize the vitamin as long cobalt source is available. The vitamin is then accumulated in the liver of those animals. Certain insects such as termites also contain vitamin B_{12} provided by the gut bacteria (Wakayama et al. 1984). On the contrary, the microflora in humans' gastrointestinal tracts is not sufficient to provide vitamin B_{12}. Hence, humans acquire the vitamin from their diet, mostly by consuming animal-derived products such as meat, eggs and dairy products, or taking vitamin B_{12} supplements.

Table 1 lists vitamin B_{12} content in some variety of foods. Meat of ruminants contains higher vitamin B_{12} content than poultry. Seafood, especially oysters, clams, fish roe and octopus provide substantial amount of vitamin B_{12}. Vitamin B_{12} is generally absent in plant-derived foods, unless they are fermented, contaminated or fortified. Some good vegetarian sources of vitamin B_{12} are tempe, seaweeds and *Chlorella* extracts. Tempe, fermented soybean by *Rhizopus spp.* moulds, contains vitamin B_{12} due to contaminating bacteria such as *Citrobacter freundii* and *Klebsiella pneumoniae* (Keuth and Bisping 1994; Astuti et al. 2000). The content of vitamin B_{12} in tempe varies depending on the conditions of fermentation and the type of contaminating bacteria. Seaweeds and other edible algae are reported to contain sufficient amount of vitamin B_{12}. However, the activity of vitamin B_{12} in edible algae for humans is still in debate. Research carried out by Watanabe et al. (1999) showed that *Spirulina* tablets contains mostly pseudo-vitamin B_{12} (7-adenyl cyanocobamide), a corrinoid inactive for humans. Although others indicated that *Chlorella* and laver can provide adequate amounts of active vitamin B_{12} (Rauma et al. 1995; Takenaka et al. 2001; Kiittaka-Katsura et al. 2002).

Table 1. Vitamin B_{12} content of foods (Liem et al. 1977; Kiittaka-Katsura et al. 2002; Stabler and Allen 2004; Kwak et al. 2010).

Foods	**B_{12} (µg/100g)**	**Foods**	**B_{12} (µg/100g)**
Beef liver	83.10[a]	Egg	1.29[a]
Beef (ground)	2.56[a]	Clams	96.60[a]
Lamb	3.03[a]	Oysters	18.70[a]
Goat	1.19[a]	Herring	9.62[a]
Pork	0.75[a]	Tuna (canned)	2.99[a]
Rabbit	8.30[a]	Fish roe	10.00[a]
Turkey	0.37[a]	Octopus	36.00[a]
Chicken	0.22[a]	Anchovy	17.12[a]
Duck	0.30[a]	Tempe	0.18-6.30[a,c]
Cow milk (1%)	0.44[a]	Fish sauce[1]	1.90[c]
Cow milk (whole)	0.20-0.79[a]	Laver, dried, seasoned and toasted (Nori)	55.3-71.3[b,c]
Cow milk (evaporated)	0.25[a]	Sea lettuce, raw	84.7[a,c]
Cheese (cheddar)	2.93[a]	Chlorella tablets	200[b,c]

[a] Vitamin B_{12} content in µg/100g fresh weight
[b] Vitamin B_{12} content in µg/100g dry weight
[c] Reported vitamin B_{12} content probably includes analogues

2.4 Physiological Functions of Vitamin B_{12}

2.4.1 Metabolic Processes in Humans

In mammalian cells, vitamin B_{12} is essential to assist two enzymatic activities in protein metabolism: methionine synthase and (R)-methyl-malonyl-CoA mutase (Figure 2). After absorption, instrinsic factor, haptocorrin (R-protein or transcobalamin I) and transcobalamin II (Nielsen et al. 2012) as carrier proteins bind vitamin B_{12} and transport it to the two enzymes. Methionine synthase or homocysteine methyltransferase (in the cytosol) requires vitamin B_{12} in the form MeCbl to methylate homocysteine to form methionine using 5-methyltetrahydrofolate as a methyl donor. When vitamin B_{12} is deficient, accumulation of 5-methyltetrahydrofolate occurs, resulting in diminished tetrahydrofolate (THF) that is specifically required to convert deoxyuridylate to thymidylic acid, one of the four essential precursors in DNA synthesis.

(R)-methyl-malonyl-CoA mutase (in the mitochondria) requires AdoCbl as coenzyme in the catabolism of propionyl-CoA, which is degraded from odd-chain fatty acids, certain amino acids (thymine, valine, methionine, threonine and isoleucine) and cholesterol. In this process, propionyl-CoA is carboxylated to (S)-methylmalonyl-CoA and epimerized to form the (R)-isomer. (R)-methyl-malonyl-CoA mutase with AdoCbl converts the isomer to succinyl CoA that ends up in the tricarboxylic acid (Krebs) cycle (Martens et al. 2002; Truswell 2007).

Together with pyridoxine (vitamin B_6) deficiency and folate deficiency, vitamin B_{12} deficiency also leads to elevated homocysteine; a junction metabolite that synthesizes intracellular antioxidant glutathione (GSH) (McCaddon et al. 2002; Birch et al. 2009). Increased homocysteine level, a condition called hyperhomocysteinemia, is associated with endothelial cell dysfunction. It also prevents the antioxidant enzymes superoxide dismutase and glutathione peroxidase and triggers endothelial proinflammatory signaling pathways. These problems are represented by a major risk for heart disease, atherosclerosis, stroke and vascular disease.

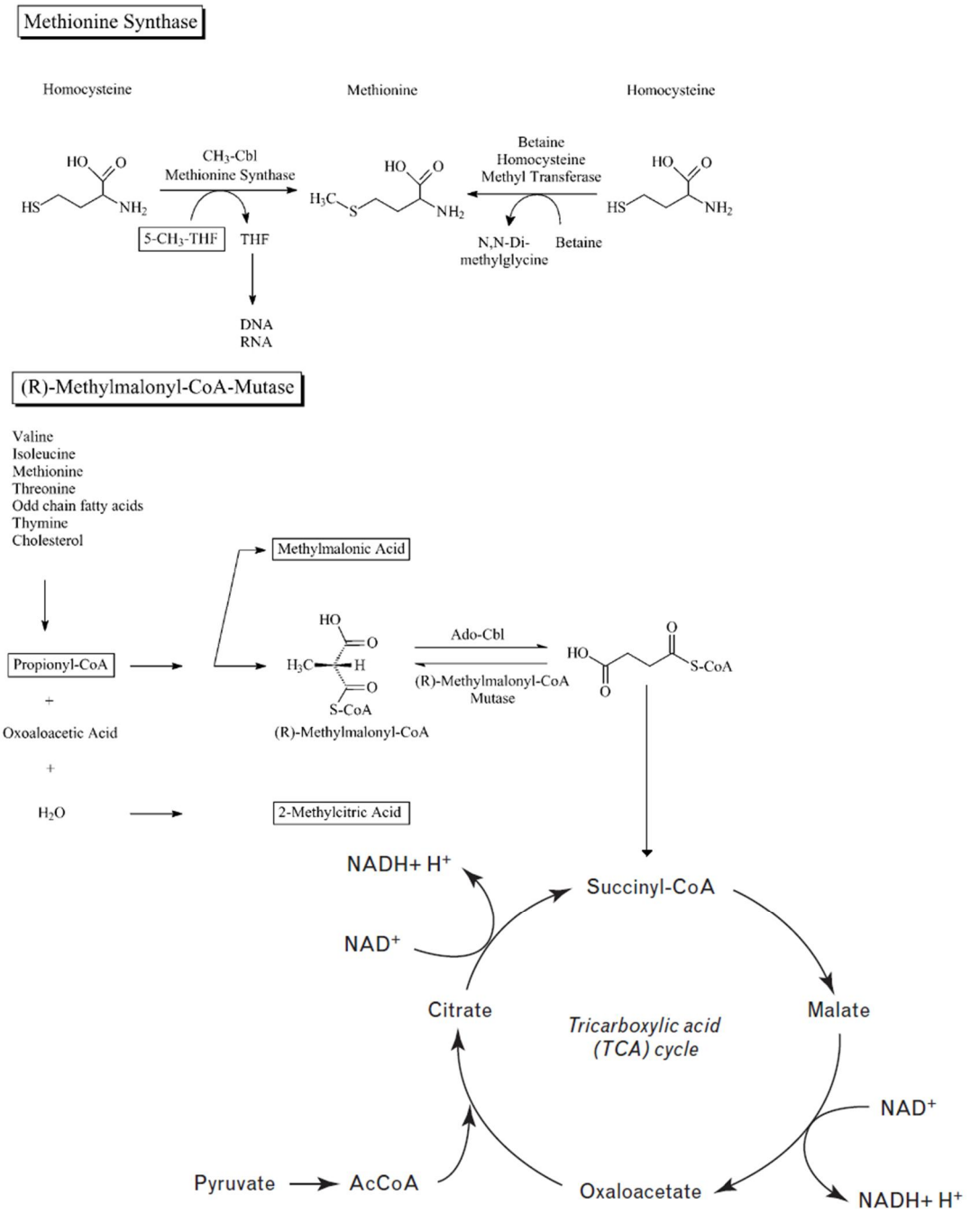

Figure 2. Cobalamin-dependent enzymatic metabolisms in humans and related metabolic pathways (Martens et al. 2002; Manzanares and Hardy 2010).

2.4.2 Transport and Absorption of Vitamin B_{12}

Transport and absorption of vitamin B_{12} from food to the body's cells are as sophisticated as its structure, requiring multistep pathways. Through genetic, biochemical and clinical studies on patients with inborn errors of cobalamin metabolism, more than 15 gene products (some are listed in Table 2) are recently acknowledged to take part in these pathways. The uptake and transport of vitamin B_{12} (Figure 3) begin with three homologous carrier proteins in extracellular fluids:

haptocorrin, intrinsic factor, and transcobalamin. Each of these proteins is able to bind a single vitamin B_{12} molecule. In the upper gastrointestinal tract, food-bound cobalamin is released by peptic activity. Subsequently, haptocorrin that presents in saliva, breastmilk and gastric fluids, binds the released vitamin B_{12} and transport it to the small intestines (Quadros 2009). Haptocorrin is thought to protect vitamin B_{12} from hydrolysis due to the acidic condition in the stomach. In the duodenum, haptocorrin is degraded by pancreatic enzymes and vitamin B_{12} is bound by instrinsic factor secreted by parietal cells of the stomach. In the terminal ileum, intrinsic-factor-bound vitamin B_{12} is bound on the apical surface of ileal epithelial cells through endocytosis by cubam (Fyfe et al. 2004); a receptor complex consisting of cubilin and amnionless (AMN; a transmembrane, endocytic protein). Inside the enterocyte, intrinsic factor is degraded in the lysosome by proteases. Assisted by protein LMBD1, the freed vitamin B_{12} then passes the lysosomal membrane to enter the cytoplasm.

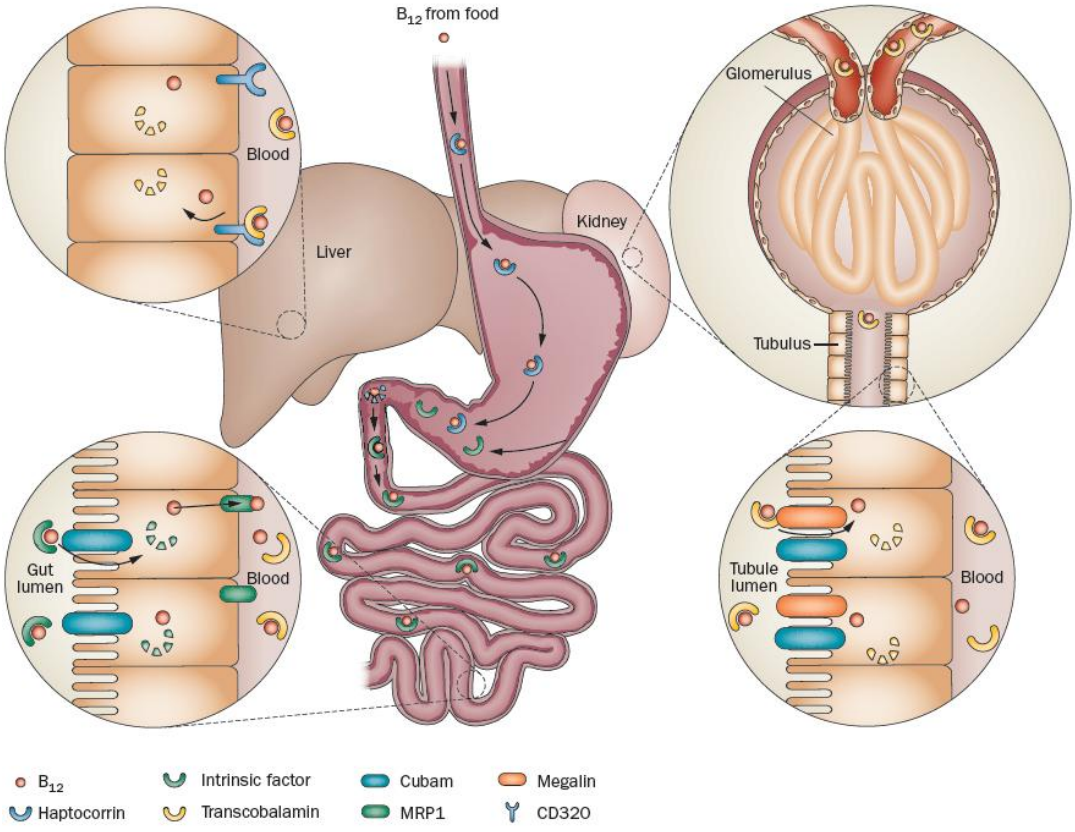

Figure 3. Uptake and transport pathways of vitamin B_{12} in humans (Nielsen et al. 2012).

The exact metabolism of vitamin B_{12} from lysosomal exit until its usage as a coenzyme and cofactor is however still slightly known. Based on the current knowledge, Figure 4 illustrates the pathways of vitamin B_{12} cellular uptake and exit. In the cytoplasm, vitamin B_{12} is bound by the cobalamin C protein then passed on to

the cobalamin D protein that is suggested to carry vitamin B_{12} to apo-methionine synthase in the cytosol and to apomethylmalonyl-CoA mutase in the mitochondrion (Nielsen et al. 2012). Inside the mitochondrion, cobalamin B protein modifies the vitamin B_{12} fraction to generate AdoCbl, which is later used by (R)-methyl-malonyl-CoA mutase. The function of the cobalamin A protein is suggested to ensure AdoCbl remains in adenosylated state. In the cytosol, the cobalamin E protein is thought to generate the rest of vitamin B_{12} to MeCbl that is used by methionine synthase (Froese and Gravel 2010).

Besides assisting two enzymatic activities, vitamin B_{12} can exit the cell to enter extracellular fluid or bloodstream. The remaining B_{12} enters plasma from the basolateral membranes passing through the ATP-binding cassette (ABC) transporter, MRP1 (Beedholm-Ebsen et al. 2010). In the bloodstream, vitamin B_{12} is bound to transcobalamin, which delivers vitamin B_{12} to cells of peripheral tissues. The transcobalamin-bound-B_{12} is then taken up by cells in the liver and other tissues with the help of an endocytic receptor, CD320 protein. Through renal filtration, some of the transcobalamin-bound-B_{12} is also reabsorbed in the kidney mediated by the receptor megalin (Figure 3), a protein belongs to LDL receptor family located on the apical membrane of proximal tubule cells (Birn et al. 2002).

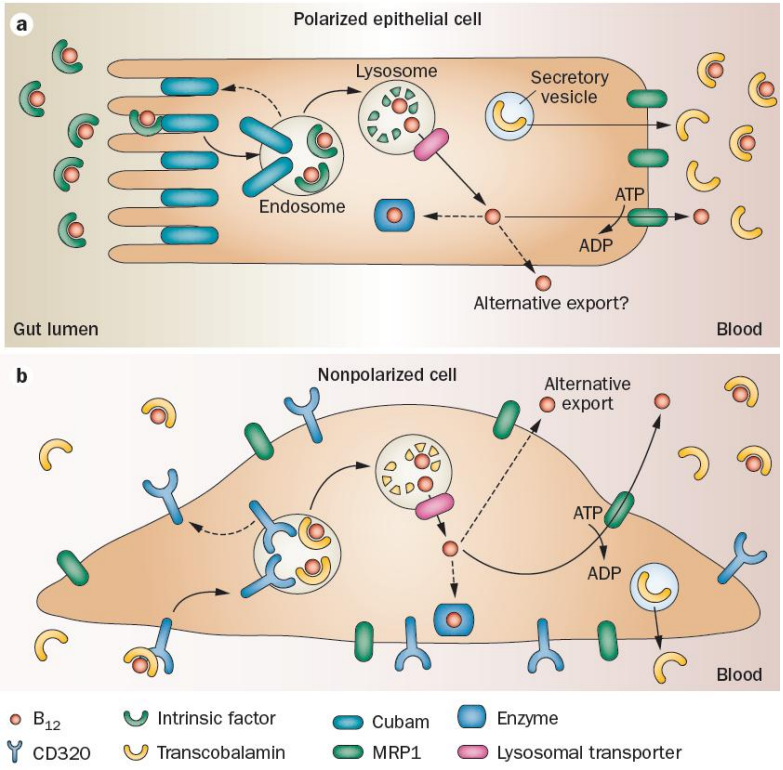

Figure 4. Pathways of vitamin B_{12} cellular uptake and exit (Nielsen et al. 2012)**.**

Table 2. Gene products involved in vitamin B_{12} transport and absorption from food to cells (Banerjee et al. 2009; Froese and Gravel 2010; Nielsen et al. 2012).

Gene product	Location of function	Function in vitamin B_{12} trafficking
Haptocorrin	Body fluids	To bind vitamin B_{12}
Intrinsic factor	Small intestine	To bind vitamin B_{12}
Transcobalamin	Blood	To bind vitamin B_{12}
Cubilin (CUBN)	Apical surface of the brush border epithelial cells in the terminal ileum	The intrinsic factor-B_{12}-binding subunit of cubam
Amnionless (AMN)	Apical surface of the brush border epithelial cells in the terminal ileum	The transmembrane cubam subunit that accounts for internalization of vitamin B_{12}
Cobalamin A protein (MMAA)	Mitochondrion	To ensure that the cofactor form bound by methylmalonyl-CoAmutase is adenosylcobalamin
Cobalamin B protein (MMAB)	Mitochondrion	To catalyze adenosylation of vitamin B_{12}
Cobalamin C protein (MMACHC)	Cytosol	To catalyze decyanation of cyanocobalamin and dealkylation of alkylcobalamins
Cobalamin D protein (MMADHC)	Cytosol	To bind intracellular vitamin B_{12} and direct it to the mitochondrial or cytosolic pathway
Cobalamin E protein (MTRR)	Cytosol	To catalyze vitamin B_{12} methylation
Cobalamin F protein (LMBD1)	Lysosomal membrane	To transport vitamin B_{12} from the lysosome to the cytosol
Methionine synthase/cobalamin G protein	Cytosol	To catalyze methylation of homocyteine to form methionine
(R)-methyl-malonyl-CoA mutase (MUT)	Mitochondrion	To catalyze the conversion of methylmalonyl-CoA to succinyl-CoA
CD320	Plasma membrane	To bind transcobalamin-B_{12}
Multidrug resistance protein 1 (MRP1/ABCC1)	Basolateral membranes	Molecular gateway for vitamin B_{12} cellular export.

2.5 Deficiency of Vitamin B_{12}

Vitamin B_{12} deficiency and depletion commonly occur worldwide, particularly among vegetarians, vegans and the elderly (Matthews 1995; Stover 2010). This prevalence is higher in developing nations and poorer populations in which people have a low intake of animal products. The deficiency is usually detected by low serum vitamin B_{12} concentration, measured by microbiological or radio assay. A patient is diagnosed with vitamin B_{12} deficiency when serum B_{12} is <148 pmol/L. While marginal depletion is defined with serum B_{12} is 148-221 pmol/L (Allen 2009). Other indicators of vitamin B_{12} deficiency are elevated serum methylmalonic acid (MMA>210 nmol/L) and homocysteine. Raised serum MMA provides more reliable vitamin B_{12} deficiency status than homocysteine as serum MMA is not increased by folate deficiency (Truswell 2007).

2.5.1 The Nutritional Requirements

To achieve absorption of 1 µg/d, the Institute of Medicine (1998) set Recommended Daily Allowance (RDA) of vitamin B_{12} at 2.4 µg/d, assumed that 50% of dietary vitamin B_{12} is absorbed by healthy adults (Heyssel et al. 1966; Russell et al. 2001). The required intake is higher for pregnant and lactating women; 2.6 µg/day and 2.8 µg/day, respectively. Consumption of vitamin B_{12} fortified foods or vitamin B_{12}-containing supplements is highly recommended for those older than 50 years as 10-30% of older adults may be unable to absorb naturally occurring vitamin B_{12}. Vitamin B_{12} has a low risk of toxicity, even taken in high doses and the upper limits for intake have not been defined (Flynn et al. 2003). Table 3 lists RDA of vitamin B_{12} based on age groups.

Table 3. Recommended dietary allowance for different age groups.

Age	Food and Nutrition Board[1](µg/d)	FAO/WHO[2] (µg/d)
0 to 6 months	0.4	0.4
7 to 12 months	0.8	0.7
1 to 3 years	0.9	0.9
4 to 8 years	1.2	1.2 (4 to 6 years)
		1.8 (7 to 9 years)
9 to 13 years	1.8	2.4
14 to 18 years	2.4	2.4
>19 years	2.4	2.4
Pregnant women	2.6	2.6
Lactating women	2.8	2.8

[1] Food and Nutrition Board (2000)
[2] FAO/WHO (2004)

2.5.2 Deficiency Diseases

Since the liver is able to store sufficient amount of vitamin B_{12} for several years, vitamin B_{12} deficiency is developed gradually. Thus, biochemical and subclinical deficiency, i.e. low B_{12} serum and/or high MMA appear first. Two major clinical syndromes: megaloblastic anaemia and/or neuropathy occur in the severe deficiency (serum B_{12}<120-150 pmol/L). In megaloblastic anaemia, immature nuclei, enlarged red cells (macrocytosis) and hypersegmentation in granulocytes are found in peripheral blood and bone marrow (Stabler 2013; Truswell 2007). The neuropathy symptoms include spinal cord, cranial or peripheral nerve, or cerebral demyelination, which may lead to muscle weakness, spasticity, dementia, psychoses, ataxia, and Alzheimer's disease. Infants of vitamin B_{12}-deficient or vegan mothers may be born with the deficiency or it develops if they are exclusively breastfed. Symptoms in infants occur between 4-6 months of age and these include movement disorders (tremors, twitching and writhing), abnormal reflexes, feeding difficulties, hyperirritability, lethargy, developmental regression and failure of brain growth that can progress to coma (Honzik et al. 2010). Early diagnosis and immediate vitamin B_{12} replacement can reverse the symptoms and prevent permanent disabilities.

2.5.3 Causes of Vitamin B_{12} Deficiency

In younger adults and developing countries, inadequate dietary intake is likely the main cause of vitamin B_{12} deficiency while in older adults, malabsorption of the vitamin from the food is the common cause of deficiency. Table 4 lists causes of vitamin B_{12} deficiency and the recommended treatments.

Table 4. Causes and treatments of vitamin B_{12} deficiency (Yousef and Andrés 2008; Nielsen et al. 2012; Stabler 2013).

Cause	Treatment
Severe malabsorption	
Pernicious anemia (autoimmune gastritis)	1000 µg of intramuscular cyanocobalamin, injected daily or every other day for a week, then weekly 4-8 week followed by monthly injection for life. Another option is oral treatment of 1000-2000 µg cyanocobalamin, taken daily for life.
Gastric bypass surgery	
Total/partial gastrectomy	
Inflammatory bowel disease, tropical sprue	
Chrohn's disease	
Coeliac disease	
Parasitic infection e.g. fish tapeworm *Diphyllobothrium latum* and protozoa *Giardia lamblia*	Treating the infection, followed by cyanocobalamin injection.
Mild malabsorption	
Mild atrophic gastritis, related or unrelated to *Helicobacter pylori* infection	Oral treatment of 500-1000 µg daily or intramuscular cyanocobalamin of 1000 µg daily or every other day for a week, then weekly 4-8 week followed by monthly injection for life.
Protein-bound vitamin B_{12} malabsortion	
Prolonged use of antacids	
Dietary deficiency	
Breastfed infants by vitamin B_{12}-deficient mothers	Daily cyanocobalamin injection of 250-1000 µg, then weekly until full recovery. Treatment of mother to enrich breast milk; daily supplementation of 1-2 µg of vitamin B_{12} or vitamin B_{12}-enriched food.
Practicing vegetarian or vegan diet, or diet low in meat and dairy products	Take supplements containing >2 µg of vitamin B_{12} or B_{12}-fortified foods.

2.6 Vitamin B_{12} Biosynthesis in Microorganisms

Biosynthesis of vitamin B_{12} exists only in microorganisms; some bacteria and archaea. Although animals (including humans) and protists require cobalamin, they are not able to synthesize it, while plant and fungi ostensibly neither require nor synthesize it. There are two biosynthesis pathways for vitamin B_{12} (Blanche et al. 1993; Burgess et al. 2009): (1) oxygen-dependent pathway (aerobic) that is found in *Pseudomonas denitrificans* and (2) oxygen-independent pathway (anaerobic), which has been shown in *Salmonella typhimurium*, *Bacillus megaterium* and *Propionibacterium freudenreichii*.

The biosynthesis of vitamin B_{12} begins with the formation of 5-aminolevulenic acid (ALA), the first general precursor of all known tetrapyrroles (Raux et al. 2000; Martens et al. 2002; Scott and Roessner, 2002). ALA is synthesized by two biosynthetic routes, C5-Way and C4-Way (Shemin-Way) (Figure 5). Plants, archaea and most bacteria synthesize ALA via C5-Way, in which the C-5 skeleton of glutamyl-tRNA is reduced to glutamate-1-semialdehyde (GSA) by NADPH-dependent glutamyl-tRNA reductase (GluTR). Later, pyridoxal 5'-phosphate (PLP)-dependent glutamate-1-semialdehyde-2, 1-aminomutase (GSAM) converts GSA into ALA by a transamination reaction (Layer et al. 2010). While mammals, fungi and α-group of proteobacteria produce ALA via Shemin-Way (named after one of the two discoverers), in which glycine and succinyl-CoA are condensated and then catalyzed by 5-aminolevulinic acid synthase (ALAS).

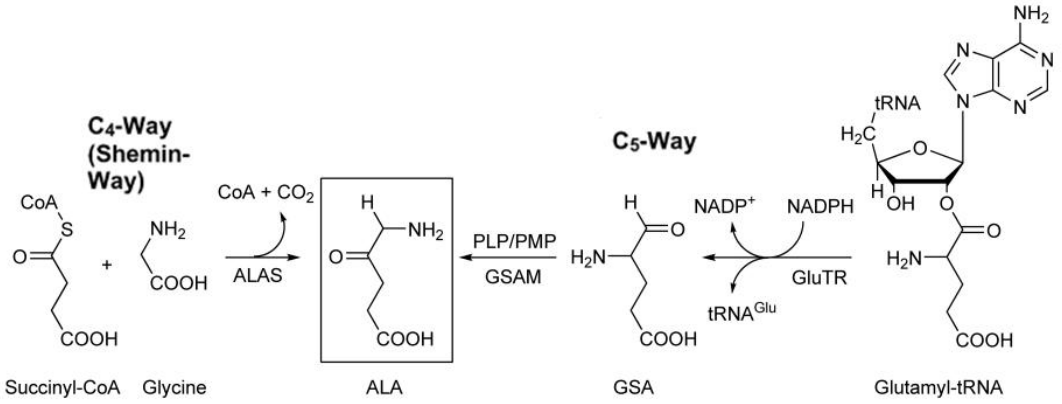

Figure 5. Formation of 5-aminolevulinic acid (ALA) via C5-Way and Shemin-Way (Layer et al. 2010).

Eight molecules of ALA then undergo three consecutive enzymatic steps to form uroporphyrinogen III (Figure 6a). Firstly, two molecules of ALA are condensed with the help of porphobilinogen synthase (PBGS) to produce the pyrrole derivative, porphobilinogen. Next, porphobilinogen deaminase (PBGD) connects four molecules of porphobilinogen to form preuroporphyrinogen (1-hydroxymethylbilane), which is then converted to uroporphyrinogen III (the first cyclic tetrapyrrole) under inversion of the D ring by uroporphyrinogen III synthase (UROS). If decarboxylated, uroporphyrinogen III is the intermediate in chlorophyll and hemes biosynthesis. In cobalamin biosynthesis, uroporphyrinogen III is instead methylated at C-2 and C-7 to form precorrin-2, a dimethylated dipyrrocorphin that is also found in siroheme, heme d_1 and coenzyme F430 synthesis (Martens et al. 2002). The methylation is catalyzed by S-adenosyl-L-methionine uroporphyrinogen III methyltransferase (SUMT).

Furthermore, cobalamin and siroheme biosynthesis are closely integrated (Figure 6b) as two intermediates in siroheme biosynthesis; precorrin-2 and sirohydrochlorin (factor II) distinguish aerobic and anaerobic pathways of cobalamin biosynthesis. The formation of adenosylcobalamin from uroporphyrinogen III in both pathways involves these seven following reactions:

- The attachment of eight methyl groups derived from S-adenosyl-L-methionine (SAM).
- The insertion of cobalt.
- Ring contraction with removal of the methylated C-20 resulting as either acetic acid or acetaldehyde.
- Decarboxylation of the acetic acid side chain on ring C.
- Amidation of six of the eight acidic side chains.
- The formation of upper axial ligand.
- Attachment of D-1-amino-propanol-2 to the propionate side chain on ring D.
- Biosynthesis and attachment of the lower nucleotide loop.

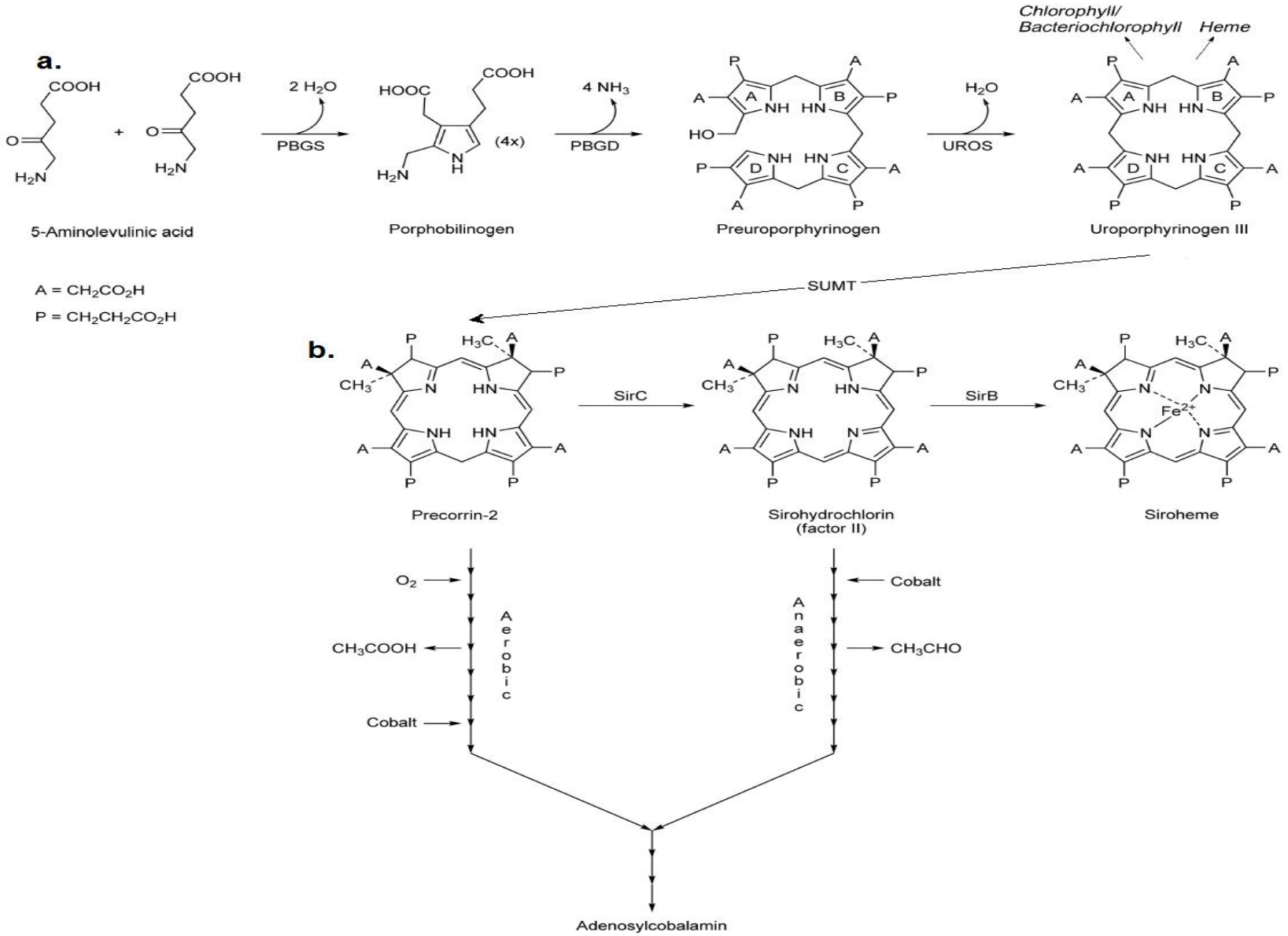

Figure 6. a. Conversion of ALA into uroporphyrinogen III by three consecutive enzymatic steps. **b.** Integration of siroheme and cobalamin biosynthesis. Modified from Martens et al. (2002) and Layer et al. (2010).

The major differences between the two pathways are the timing of cobalt insertion and the method used to promote the ring-contraction. In the anaerobic pathway, cobalt insertion occurs at early step (Figure 7a); in which sirohydrochlorin is chelated to form cobalt-precorrin-2, a reaction that is catalyzed by a number of cobaltochelatases: CbiK, $CbiX^L$, or $CbiX^S$ (Raux et al. 1997; Brindley et al. 2003; Leech et al. 2003). In the aerobic pathway, the cobalt insertion occurs after nine further reactions, where hydrogenobyrinic acid a,c-diamide is chelated to form Cob(II)yrinic acid a,c-diamide mediated by CobN, CobS and CobT (Debussche et al. 1992). In the aerobic pathway, the cobalt insertion requires ATP, while no high-energy equivalents required in the anaerobic pathway. To promote the ring-contraction process (Figure 7b), under aerobic conditions, the C-20 atom of precorrin-3A is firstly oxidized by O_2 to form precorrin-3B, a process catalyzed by a Fe_4S_4 cluster containing protein (CobG) resulting the release of C-20 as acetic acid. In the presence of SAM, Cob J then mediates the ring contraction and methylates the C-17 in precorrin-3B to form precorrin-4. Under anaerobic conditions, the C-20 atom in cobalt-precorrin-2 is methylated by CbiL resulting cobalt-factor III or cobal-precorrin-3, which is later mediated by CbiH to undergo the ring contraction. The C-20 atom in anaerobic pathway is then released as acetaldehyde.

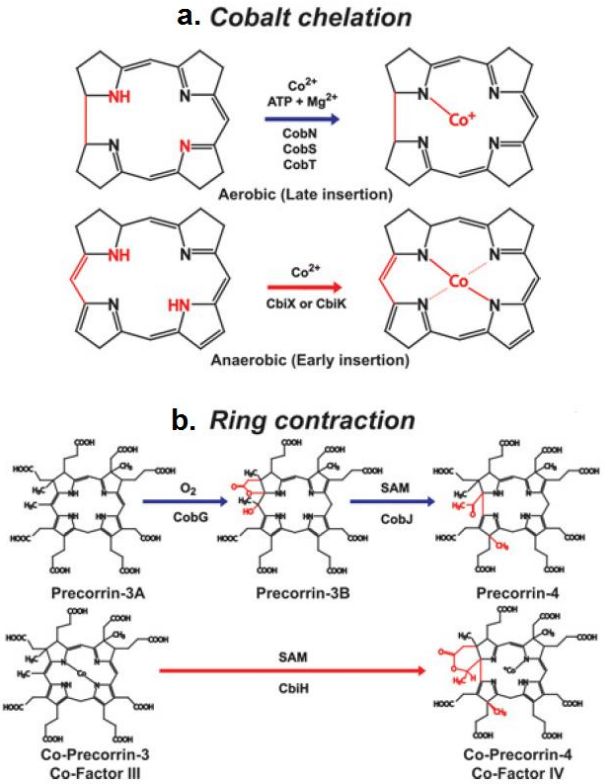

Figure 7. Simplified diagram of cobalt insertion (**a.**) and ring contraction (**b.**) in the aerobic and anerobic pathways, modified from Moore and Warren (2012)**.**

The differences are shown at the genetic level by the presence or absence of more than 30 genes involved in the biosynthesis (Table 5). The genes encoding proteins are isolated and sequenced from *Pseudomonas denitrificans* (Blanche et al. 1995), *Salmonella typhimurium* (Roth et al. 1993), *Bacillus megaterium* (Raux et al. 1998) and *Propionibacterium freudenreichii subsp. shermanii* (Roessner et al. 2002). The genes are distinguished to prefix *cob* (cobalamin biosynthesis) for the aerobic pathway and prefix *cbi* (cobinamide biosynthesis) for the anaerobic pathway. Despite these differences, at the level of adenosylcobyric acid, both pathways undergo identical steps involving conversion of Ado-cobyric acid to Ado-cobalamin by the addition of aminopropanol and DMBI via adenosylcobinamide.

Table 5. The genes listed for the oxygen-dependent pathway are from *P. denitrificans*. The genes listed for the oxygen-independent pathway are from *S. enterica*. Adapted from Roessner et al. (2002), Roessner and Scott (2006) and Layer et al. (2010).

Oxygen Dependent/Late Cobalt Insertion Pathway	**Gene**	**Oxygen Independent/Early Cobalt Insertion Pathway**	**Gene**
Aminolevulinic acid (ALA) ⇓ *condensation of 2 ALA*	*hemB*	Aminolevulinic acid (ALA) ⇓ *condensation of 2 ALA*	*hemB*
Porphobilinogen ⇓ *tetrapyrrole formation*	*hemC*	Porphobilinogen ⇓ *tetrapyrrole formation*	*hemC*
Hydroxymethylbilane ⇓ *cyclisation and ring D conversion*	*hemD*	Hydroxymethylbilane ⇓ *cyclisation and ring D conversion*	*hemD*
Uroporphyrinogen III ⇓ *methylation at C-2 and C-7*	*cobA*	Uroporphyrinogen III ⇓ *methylation at C-2 and C-7*	*cysG*
Precorrin-2 ⇓ *methylation at C-20*	*cobI*	Precorrin-2 Sirohydrochlorin ⇓ *cobalt insertion*	*cysG, cbiK*
Precorrin-3 ⇓ *C-20 hydroxylation* ⇓ *γ-lactone formation*	*cobG*	Cobalt-precorrin-2 ⇓ *methylation at C-20*	*cbiL*
Precorrin-3-hydroxylactone ⇓ *C-17 methylation* ⇓ *ring contraction*	*cobJ*	Cobalt-precorrin-3 ⇓ *C-17 methylation* ⇓ *ring contraction* ⇓ *γ-lactone formation*	*cbiH*
Precorrin-4 ⇓ *C-11 methylation*	*cobM*	Cobalt-precorrin-4 ⇓ *C-11 methylation*	*cbiF*
Precorrin-5 ⇓ *C-1 methylation* ⇓ *acetic acid extrusion*	*cobF*	Cobalt-precorrin-5A ⇓ *opening of lactone ring* ⇓ *acetaldehyde extrusion* Cobalt-precorrin-5B ⇓ *C-1 methylation*	*cbiG* *cbiD*
Precorrin-6 ⇓ *C-18/C-19 reduction*	*cobK*	Cobalt-precorrin-6 ⇓ *C-18/C-19 reduction*	*cbiJ*
Dihydroprecorrin-6 ⇓ *C-5, C-15 methylation* ⇓ *decarboxylation*	*cobL* *cobL*	Cobalt-dihydroprecorrin-6 ⇓ *C-5, C-15 methylation* ⇓ *decarboxylation*	*cbiE* *cbiT*

Precorrin-8		Cobalt-precorrin-8	
⇓ *methyl rearrangement*	*cobH*	⇓ *methyl rearrangement*	*cbiC*
Hydrogenobyrinic acid		Cobyrinic acid	
⇓ *a,c-amidation*	*cobB*	⇓ *a,c-amidation*	*cbiA*
Hydrogenobyrinic acid a,c-diamide		Cob(II)yrinic acid a,c-diamide	
⇓ *cobalt insertion*	*cobN, cobS, cobT*	⇓ *Cobalt reduction*	*fldA*
Cob(II)yrinic acid a,c-diamide			
⇓ *Cobalt reduction*	*cobR?*		
Cob(I)yrinic acid a,c-diamide		Cob(I)yrinic acid a,c-diamide	
⇓ *adenosylation*	*cobO*	⇓ *adenosylation*	*cobA*
Ado-cob(I)yrinic acid a,c-diamide		Ado-cob(I)yrinic acid a,c-diamide	
⇓ *b,c,e,g-amidation*	*cobQ*	⇓ *b,c,e,g-amidation*	*cbiP*
Ado-cobyric acid		Ado-cobyric acid	
⇓ *aminopropanol attachment*	*cobC, cobD, ?*	⇓ *aminopropanol attachment*	*cbiB,?*
Ado-cobinamide		Ado-cobinamide	
⇓ *phosphorylation*	*cobP*	⇓ *phosphorylation*	*cobU*
⇓ *GMP addition*	*cobP*	⇓ *GMP addition*	*cobU*
Ado-GDP-cobinamide		Ado-GDP-cobinamide	
⇓ *a-ribazole addition*	*cobV*	⇓ *a-ribazole addition*	*cobS*
Ado-cobalamin 5'-phosphate		Ado-cobalamin 5'-phosphate	
⇓ *dephosphorylation*	*?*	⇓ *dephosphorylation*	*cobC*
Ado-cobalamin		Ado-cobalamin	

2.7 Biosynthesis of 5,6-dimethylbenzimidazole (DMBI)

Although the attachment of the lower nucleotide loop of cobalamin has been elucidated (Maggio-Hall and Escalante-Semerena 1999), the biosynthesis of DMBI, the lower ligand of vitamin B_{12} has remained an enigma. Previous studies on the biosynthesis of DMBI have been performed in the obligate anaerobe, *Eubacterium limosum*, in which DMBI was synthesized from formate, glycine, glutamine, SAM, and erythrose-4-phosphate (Höllriegl et al. 1982; Munder et al. 1992). It was also found that *E. limosum* efficiently transformed 5-hydroxybenzimidazole and 5-hydroxy-6-methylbenzimidazole to DMBI (Renz et al. 1993; Schulze et al. 1998). However, other studies revealed that some organisms required molecular oxygen to synthesize DMBI. *P. freudenreichii* (Renz 1970; Renz and Weyhenmeyer 1972; Hoerig and Renz 1977) and *Salmonella enterica* (Keck et al. 1998) transform riboflavin to DMBI through riboflavin 5'-phosphate/flavin mononucleotide (FMN) via an oxygen-dependent reaction (Figure 8a), in which the C-1 carbon of the ribose moiety of FMN is transformed into C-2 carbon of DMBI. In anaerobic conditions, *S.*

enterica does not synthesize DMBI; instead it synthesizes adenyl and 2-methyladenylcobamide (Keck and Renz 2000). Despite the understanding that the transformation of riboflavin to DMBI involves molecular cannibalization, the genes or enzymes involved in either the aerobic or anaerobic biosynthesis of DMBI remained unknown until the recent discovery of *bluB*, a gene identified as causing vitamin B_{12} deficiency in the phototropic purple nonsulfur bacterium, *Rhodobacter capsulatus*.

BluB gene was first found in a cluster of sequenced *R. capsulatus* genes (Pollich and Klug 1995). It was suggested that five (*bluFEDCB*) out of eight genes isolated were involved in the late steps of cobalamin biosynthesis due to the strong homology with 22 *cob* genes of *P. denitrificans*. The correction assay also indicated that *bluB* was involved in the conversion of cobinamide to cobalamin. The *blu* prefix is given due to the fact that *bluE* and *bluB* genes are required to make an aerobic culture blush after the reduction of the partial O_2 pressure (pO_2). The evidence that *bluB* was involved in DMBI synthesis first appeared in a patent (Blanche et al. 2000) using a mutant of *R. capsulatus*, which claimed that overproduction of *bluB* led to increased levels of DMBI. Later, the role of *bluB* gene in DMBI synthesis was also confirmed by Campbell et al. (2006) using mutants of *Sinorhizobium meliloti*.

According to the protein sequence, *bluB* has ≈16% similarity to several enzymes of NADH/FMN-dependent nitroreductase/oxidoreductase family. Based on this sequence, Taga et al. (2007) described the structure of *bluB* (Figure 8c) and proposed that in the presence of molecular oxygen, *bluB* is able to catalyze the cannibalism of FMN to DMBI with the release of D-erythrose 4-phosphate. An alternative mechanism of FMN transformation to DMBI was suggested by Ealick and Begley (2007) based on the simplicity of the *bluB* active site, in which a series of reactions with water dismantles the flavin after it has reacted with molecular oxygen (Figure 8b). First, FMN reacts with oxygen to yield flavin hydroperoxide followed by a ring expansion with loss of a water molecule to produce intermediate A. This disintegrates further via four hydrolysis reactions, in which it is attacked by water molecules resulting diaminobenzene. Lastly, diaminobenzene undergoes two oxidation reactions forming DMBI.

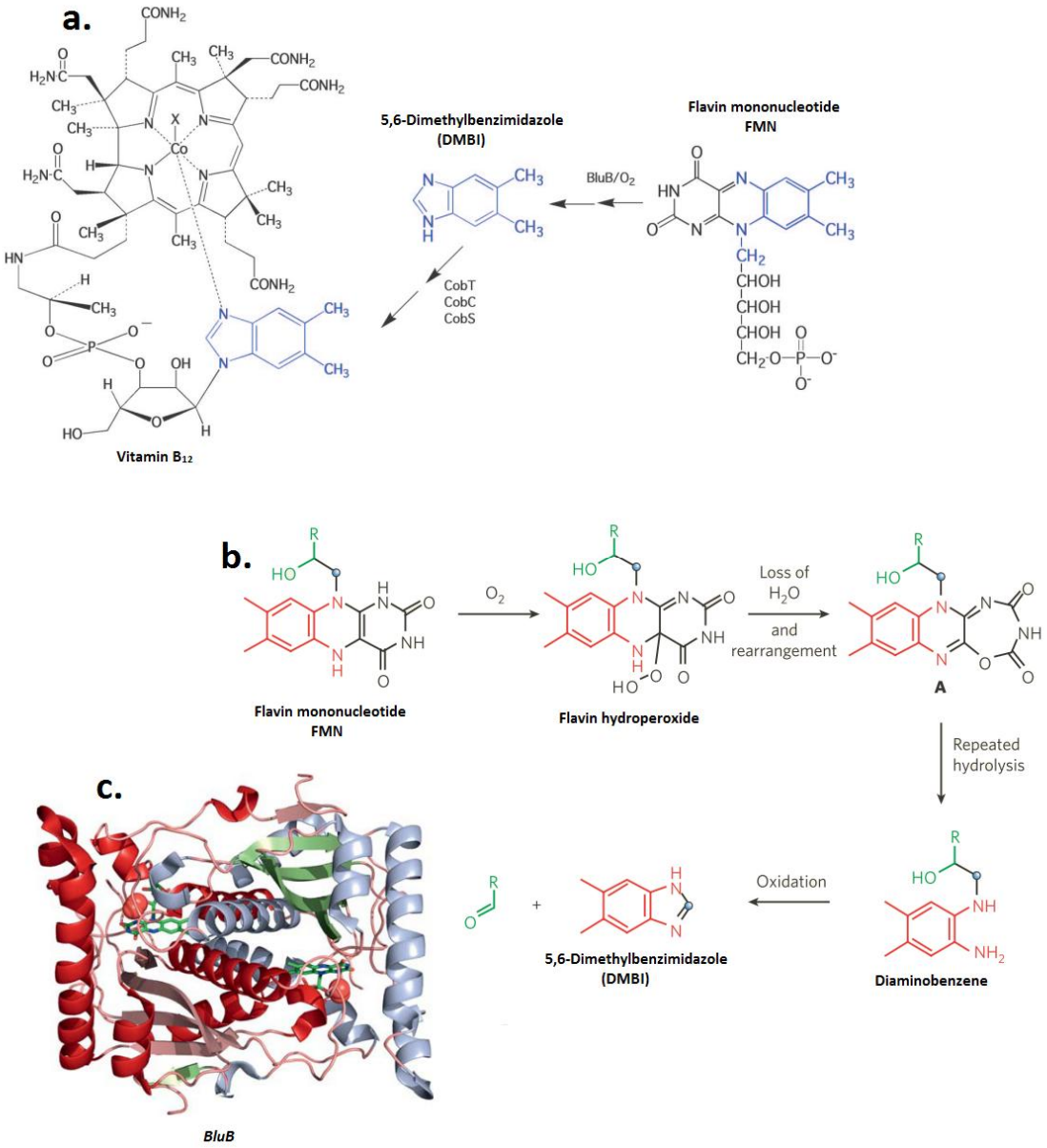

Figure 8. a. Biosynthesis of DMBI from flavin mononucleotide (FMN) in oxygen dependent reaction. (Warren 2006). **b.** Proposed mechanism that *bluB* catalyzes a reaction in which FMN transforms into DMBI (Ealick and Begley 2007). **c.** Structure of *bluB*, where the monomers are colored green helices/orange sheets and blue helices/purple sheets. The orange balls are oxygen-trapped structure of the enzyme (Layer et al. 2010).

2.8 Vitamin B_{12} Production

Chemical synthesis of vitamin B_{12} was successfully achieved by Woodward (1973) and Eschenmoser (1974). However, the chemical process requires 70 complex steps that make any industrial production by chemical methods technically too complicated and expensive. Thus, commercial vitamin B_{12} has been produced intra- or extracellularly on an industrial scale using the batch or fed-batch process of microbial fermentation (Yongsmith et al 1982), followed by extraction and purification using

organic solvents. Nowadays, the industrial production of vitamin B_{12} utilizes selected and genetically modified microorganisms to provide more economical process and enhance the quality of end products. The common methods to increase the yields of vitamin B_{12} are random mutagenesis and genetic engineering. In random mutagenesis, the microorganisms are usually treated with mutagenic agents such as UV light, ethyleneimine, nitrosomethyluretane or N-methyl-N'-nitro-N-nitrosoguanidine. The strains are then selected according to the practical advantages such as genetic stability, productivity, reasonable growth rates and resistance to high concentrations of toxic precursors present in the medium (Martens et al. 2002).

There are currently three bacteria used for commercial production of vitamin B_{12}: *Bacillus megaterium*, *Pseudomonas denitrificans*, and *Propionibacterium* (Battersby 1994; Roth et al. 1996; Hunik 1999), the latter two are more commonly used today due to the high yields. Since the strains from genus *Propionibacterium* are food-grade and produce no exotoxins or endotoxins, they are preferably used and granted GRAS (generally recognized as safe) by the United States Food and Drug Administration. Thus, vitamin B_{12} can be directly produced in the food or added to the food, without any further complex processing. Nonetheless, one of the biggest vitamin B_{12}-producing companies from France, Rhône-Poulenc, now known as Sanofi-Aventis, uses mainly *P. denitrificans* to produce the vitamin. Under Rhône-Poulenc, Blanche et al. (1995) created a highly effective vitamin B_{12}-producing strain from genetically engineered *P. denitrificans*. Although no official information is known on the productivity, the strain is believed to have productivity up to 300 mg/L. An article made by Yang et al. (2003) reported the use of methanogens in vitamin B_{12} production that have several advantages over conventional vitamin B_{12} producers such as (1) ten times higher vitamin B_{12} concentration in the broth than in *Propionibacterium* (Zhang et al. 2001); (2) methane does not inhibit the growth of methanogens and could provide a high cell density culture system; and (3) methanol, CO_2 and acetic acid used as substrates are inexpensive, relatively stable and renewable. More vitamin B_{12} producing species are observed from the following genera: *Aerobacter, Agrobacterium, Alcaligenes, Azotobacter, Bacillus, Clostridium, Corynebacterium, Flavobacterium, Micromonospora, Mycobacterium, Norcardia, Propionibacterium, Protaminobacter, Proteus, Rhizobium, Salmonella, Serratia, Streptomyces, Streptococcus* and *Xanthomonas* (Perlman 1959). Table 6 enlists the most active species of microorganisms utilized in vitamin B_{12} production.

Table 6. Species of microbial producers recommended for vitamin B_{12} production. (Bykhovsky et al. 1998)

Species	Substrate	Condition of fermentation	Yield (mg/L)
Propionibacterium freudenreichii	Glucose	Anaerobiosis, DMBI	206.0
Rhodopseudomonas protamicus	Glucose	DMBI	135.0
Propionibacterium shermanii	Glucose	DMBI	60.0
Pseudomonas denitrificans	Sucrose	Aerobiosis, betaine	60.0
Nocardia rugosa	Glucose	Aerobiosis	18.0
Rhizobium cobalaminogenum	Sucrose	Aerobiosis	16.5
Micromonospora sp.	Glucose	DMBI	11.5
Streptomyces olivaceus	Glucose	DMBI	6.0
Nocardia gardneri	Hexadecane	Aerobiosis	4.5
Butyribacterium methylotrophicum	Methanol	Anaerobiosis	3.6
Pseudomonas sp.	Methanol	DMBI	3.2
Arthrobacter hyalinus	Isopropanol	DMBI	1.1

2.8.1 Fermentative Production of Vitamin B_{12}

Previous descriptions about fermentative production of vitamin B_{12} by *Propionibacterium* have mainly focused on growing the bacteria to high cell densities by varying the nutrient composition of the growth medium such as amino acid or mineral composition, which apparently only affected vitamin B_{12} production when the growth yield of the *Propionibacterium* was improved (Hugenholtz and Smid 2002). It was later found that both the addition of the precursor DMBI and aerobic incubation in the latter phase of fermentation increased vitamin B_{12} yields. Based on these two experimental findings, Hunik (1999) patented a semi-continuous, two-stage, large-scale production process that resulted in a 3-fold increase of vitamin B_{12} production.

Albeit *Propionibacterium* can grow under aerobic conditions, the production of corrinoids (precursors of vitamin B_{12}) is absent when the concentration of dissolved oxygen is above 0.19 mM (6 mg O_2/L). The lower the O_2 concentration, the higher the corrinoid production with a maximum production under non-aerated conditions (Quesada-Chanto et al. 1998). In contrary, the biosynthesis of DMBI requires oxygen. Thus, the production using *Propionibacterium* strains is divided into two stages. In the first stage, the bacteria are grown anaerobically for 72-88 hours to produce cobamide, a precursor of vitamin B_{12} missing the DMBI moiety (Martens et al. 2002). In the second stage, aeration is applied for another 72-88 hours, allowing the bacteria to undertake the aerobic synthesis of the DMBI and to link it to cobamide. The

fermentation also produces propionic acid, which gives advantage in inhibiting the growth of other microorganisms such as lactic acid bacteria and yeasts. However, at level 20-40 g/L (Hunik 1999), propionic acid starts to inhibit the growth of *Propionibacterium*. Hence, it is crucial to neutralize the accumulated propionic acid to maintain the production culture at pH 7. Contrary to the fermentation process in *Propionibacterium*, vitamin B_{12} production in *P. denitrificans* parallels aerobic growth with high vitamin B_{12} production rates if the culture is directly supplemented with DMBI and cobalt salts. The culture is aerated for about 48-72 hours at 30°C and pH values are maintained at 6-7.

2.8.2 Optimization and Genetic Engineering in Vitamin B_{12} Production

Vitamin B_{12} production can be optimized by varying the substrates, culture inoculations (Wyk et al. 2011), pH value, temperature and aeration (Quesada-Chanto et al. 1994). Besides the addition of cobalt and DMBI, further additions of potential precursors like glycine, threonine, δ-aminolevulinic acid, betaine and choline proved to be beneficial (Marwaha et al. 1983). Through cloning and expression of 10 genes belonging to the *hem*, *cob* and *cbi* from *Rhodobacter sphaeroides* and *P. freudenreichii*, Piao et al. (2004) successfully increased the production of vitamin B_{12} in *P. freudenreichii*. The group achieved 2.2-fold vitamin B_{12} concentration (1.7 mg/L) by using a recombinant *P. freudenreichii* clone that harbored an exogenous gene, *hemA*, from *Rhodobacter sphaeroides* and endogenous *hemB* and *cobA* genes. The genetic engineering in *P. denitrificans* involved the amplification of the eight genes in the *cobF-cobM* operon, which resulted a 30% increase. An additional 20% enhancement was achieved by increasing *cobA* and *cobE* genes (Blanche et al. 1998).

2.8.3 Recovery of Vitamin B_{12}

The recovery of vitamin B_{12} (Figure 9) requires several steps to obtain final product with high purity. The biomass is centrifuged to obtain a cell mass concentrate, which is later dried. The whole batch can also be concentrated or spray dried. The cell mass is heated in aqueous solution at 80-120°C for 10-30 min. at pH 6.5-8.5 to release vitamin B_{12}, which are later converted to cyanocobalamin by the addition of potassium cyanide or thiocyanate (Spalla et al. 1989), usually in the presence of sodium nitrite and heat. For animal feed additives, the vitamin solution is clarified by filtration or treatment with zinc chloride, and then precipitated by the addition of

tannic acid or cresol giving 80% of purity. For food and pharmaceutical purposes, which require higher purity, after filtration, the solution is extracted with organic solvents, such as carbon tetrachloride and later water/butanol. The last step is crystallization of vitamin B_{12} after the addition of phenol and water.

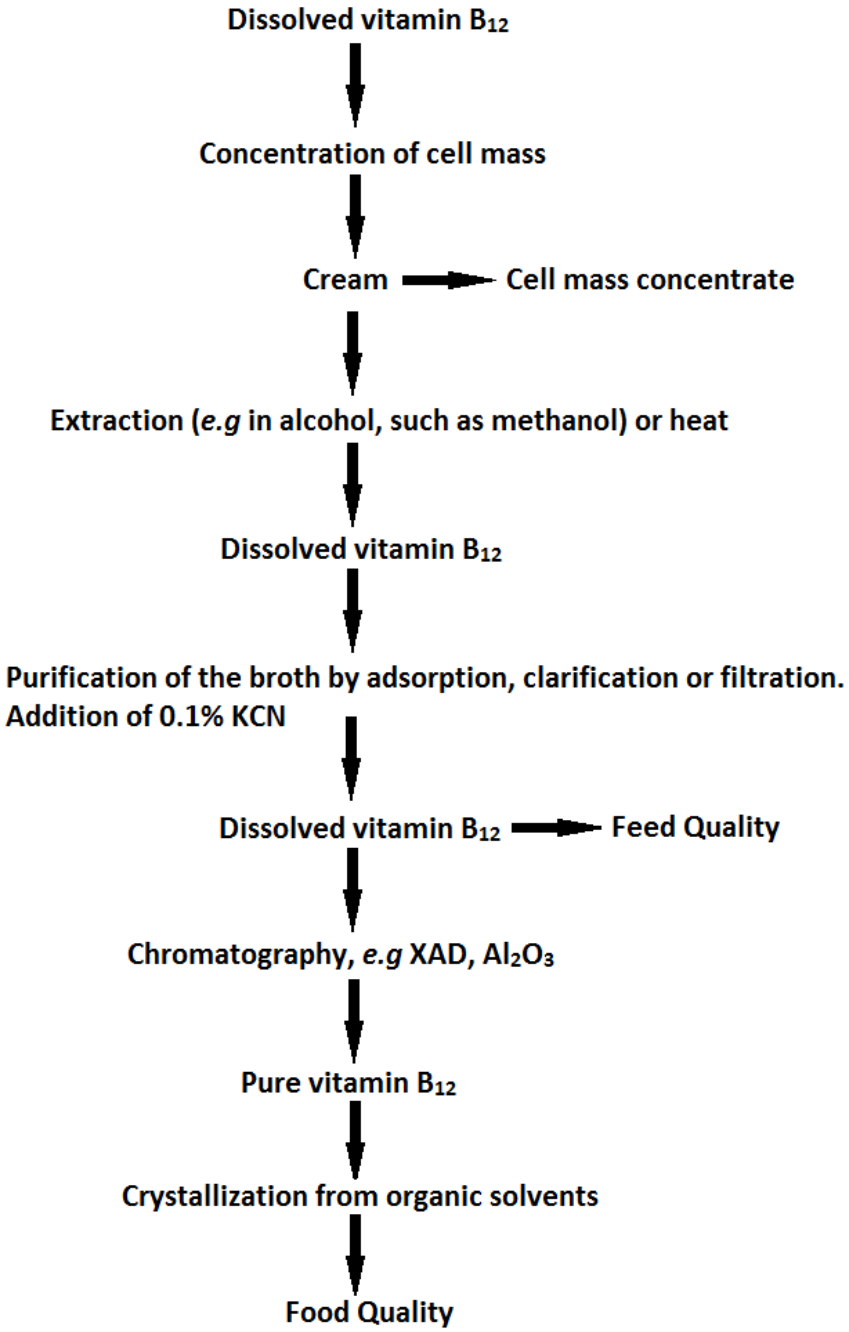

Figure 9. Flowchart of vitamin B_{12} recovery processes (Survase et al. 2006).

2.9 Production of Vitamin B_{12} in Plants-derived Foods and Spent Media

Since vegetarians and vegans are the most susceptible groups in vitamin B_{12} deficiency, as previously mentioned, they usually consume vitamin B_{12}-fortified foods and/or vitamin B_{12} supplement to meet the daily requirement. However, with the growing awareness of well-being and healthy living, the trends of consuming natural and food grade supplements are increasing. In recent vitamin B_{12} production, more researches have been carried out to optimize *in situ* cereal fermentations utilizing particularly lactic acid bacteria (LAB) due to their qualified presumption of safety (QPS) and GRAS status. Although *P. denitrificans* and *P. freudenreichii* are the most commonly used strains in vitamin B_{12} production, some LAB have been found to be vitamin B_{12} producers such as *Lactobacillus reuteri* CRL1098 (a strain isolated from sourdough), the first documented vitamin B_{12}-producing LAB (Taranto et al. 2003; Santos 2008). Moreover, the complete vitamin B_{12} biosynthesis gene cluster of *Lb. reuteri* CRL1098 has been encoded, which brings a major advantage in metabolic engineering for transferring the ability to produce vitamin B_{12} to other bacteria (Santos et al. 2008). Another LAB found in traditionally fermented sourdough, *Lactobacillus sanfranciscensis*, may open new and attractive ways for vitamin B_{12} production in cereal fermentation. Previously acknowledged of unable to synthesize folate, thiamine, riboflavin and vitamin B_6, the recently sequenced *Lb. sanfranciscensis* TMW1.1304 revealed one gene (cobyrinic acid a, c-diamide synthase, EC 6.3.5.11; LSA_2900630) involved in cobalamin synthesis (Vogel et al. 2011). Moreover, other studies have been carried out to utilize waste products as the substrate in vitamin B_{12} production. Cheese whey has been successfully shown to be used in vitamin B_{12} production (Bullerman and Berry 1966; Marwaha & Sethi 1984). Gardner and Champagne (2005) demonstrated the use of spent media (MRS medium) and prefermented vegetable juices obtained from the production pickled vegetables to produce *P. freudenreichii* biomass and vitamin B_{12}. This alternative process may be beneficial from economical and environmental standpoints as the fermentation may result in three commercial products: biomass, vitamin B_{12} or organic acids, which can be further utilized as starters, supplements or food preservatives, respectively.

The product that contains vitamin B_{12} through *in situ* cereal fermentation yet often overlooked is tempe/tempeh. Originally made by Central Javanese people in 1700s, tempe initially became popular due to its high protein content (20%), which is comparable with chicken (21%), beef (20%) and egg (13%) (Shurtleff and Aoyagi,

2001). As a vegetarian food shown to naturally contain vitamin B_{12}, tempe is produced by soaking, dehulling and cooking soybeans (or other legumes, seeds and cereal grains). The beans are then surface dried and inoculated with spores of *Rhizopus spp.* (*R. oligosporus*, *R. stolonifer*, *R. arrhizus*, and *R. oryzae*) forming compact patties. During the solid substrate fermentation (SSF), *Rhizopus* strains form other water soluble vitamins such as riboflavin, nicotinic acid, nicotinamide and vitamin B_6 (Keuth and Bisping 1993). The moulds, however, do not produce physiologically active vitamin B_{12}. In traditional tempe production, vitamin B_{12} is formed due to bacterial contamination that occurs during soaking and/or SSF. Particularly during soaking, bacterial acidification takes place, which avoids growth of spoilage microorganisms and creates optimum growth conditions for *Rhizopus spp*. In modern tempe production in Western countries, bacterial acidification is replaced by adding lactic acid to shorten the fermentation time. Moreover, the beans are inoculated with pure cultures of *Rhizopus*, explaining the absence of vitamin B_{12} (Liem et al. 1977).

In a screening for vitamin B_{12}-producing bacteria isolated from Indonesian tempe samples, 13 out of 33 isolates were *Kl. pneumoniae* (Okada 1989). Others bacteria were also found such as *Kl. terrigena*, *Kl. planticola*, *Kl. ozeanae*, *Enterobacter cloacae* and *Escherichia coli*. Two strains from *Cit. freundii* and *Kl. pneumoniae* were found to be the best vitamin B_{12}-producing bacteria in tempe production (Keuth and Bisping 1993). However, *Cit. freundii* and *Kl. pneumoniae* strains are known to possess three known genes for enterotoxin production: the genes for Shiga-like toxin SLT IIA (Schmidt et al. 1993), for heat-labile enterotoxin LT Ih (Yamamoto and Yokota 1983), and for heat-stable enterotoxin ST Ih (Moseley et al. 1983). Nevertheless, neither *Cit. freundii* nor *Kl. pneumoniae* that was isolated from Indonesian tempe, possessed the three mentioned genes, as indicated by PCR (Keuth and Bisping 1994). Interestingly, although tempe may contain many members of the family *Enterobacteriaceae*, no reports exist about diarrhea after the consumption of tempe. Hence, the use of *Cit. freundii* and *Kl. pneumoniae* in tempe fermentation should have no negative effect on the consumers. Keuth and Bisping (1994) also observed the influence of cobalt and DMBI supplementation in vitamin B_{12} content. Both precursors significantly increased vitamin B_{12} content in tempe from 73 to 170 ng/g for *Kl. pneumoniae* and up to 290 ng/g for *Cit. freundii*. However, the incorporation of the precursors was poor, resulting low yields. In conclusion, the

addition of cobalt and DMBI is unnecessary, as the vitamin B_{12} concentration produced in tempe by either of these two strains would be sufficient to meet the daily requirement of an adult.

3 EXPERIMENTAL RESEARCH

3.1 Objectives

Plant-based fermented foods that contain naturally synthesized vitamin B_{12} are still limited. In addition, published results on production of vitamin B_{12} by *Propionibacteria* (one of the highest vitamin B_{12}-producing bacteria) in plant-based foods do not exist although it has potential applications in enriching plant-based foods with vitamin B_{12}. Furthermore, the knowledge on the role of food components that can influence the synthesis of vitamin B_{12} in cereal or other plant-based foods is lacking. Thus, the primary aim of the study was to investigate the requirement of different precursors in the biosynthesis of vitamin B_{12} in optimal media conditions and then in cereal matrices using strains of *Propionibacterium freudenreichii*. The study also investigated differences of vitamin B_{12} production among the strains of *P. freudenreichii* with respect to the added precursors.

3.2 Materials and Methods

3.2.1 *Propionibacterium* Culture Preparation

Prior to vitamin B_{12} production, three strains of *P. freudenreichii* (strain 1, 2 and 3) were first inoculated on agar plates and subcultured three times for three to four days at 30°C in supplemented whey permeate (SWP) medium. All strains were kept as frozen stocks at -80°C in glycerol.

3.2.2 Chemicals and Growth Matrices

Agar Plates

All chemicals were acquired from Merck KgaA (Darmstadt, Germany) unless otherwise stated. The agar medium used for inoculation consisted of 15 g of agar powder (Difco™ Granulated Agar, Becton, Dickinson and Company, USA), 10 g of yeast extract powder (Lab M Limited, Lancashire, United Kingdom), 5 g of tryptone (Sigma-Aldrich Chemie, Steinheim, Germany) and 14 mL of sodium lactate (60%; Sigma-Aldrich Co., St. Louis, MO, USA) in 1 L of Milli-Q water (Milli-Q Plus; Millipore S.A., France). The agar plate was prepared as follows: tryptone, yeast extracts and sodium lactate 60% were diluted in Milli-Q water with

pH adjusted to 7.3 ± 0.2 (5M NaOH). The agar powder was then added in the suspension followed by autoclaving (121°C, 15 min).

Supplemeted Whey Permeate (SWP)

The SWP medium used in sub-culturing and production of vitamin B_{12} contained final concentration of 60 g of whey permeate (demineralised whey powder; Valio Ltd, Lapinlahti, Finland), 0.1 g of Tween 80 (Sigma-Aldrich Co., St. Louis, MO, USA), 10 g of yeast extract, 0.2 g of magnesium sulphate and 0.05 g of manganese (II) sulphate in 1 L of 0.1 M potassium phosphate buffer pH 6.4. The SWP was prepared according to Hugenschmidt et al. (2010): 85.7 g/L whey powder with pH adjusted to 5.0 (5M HCl) was autoclaved (121°C, 20 min). The suspension was cooled and vacuum filtered (filter paper, Ø150 mm; Whatman International Ltd, UK). 700 mL of the filtered whey permeate was mixed with 150 mL of Mg-Mn-Tween 80 solution (2.73 g/L magnesium sulphate heptahydrate, 0.37 g/L manganese (II) sulphate monohydrate and 6.67 g/L Tween 80) and autoclaved. To limit Maillard reaction, 87 mL of 1 M potassium phosphate buffer (pH 6.6) was added to a mix of 50 mL of yeast extract (20% w/v) and 13 mL of sodium D/L-lactate syrup (60% (w/w), synthetic) and autoclaved separately. The phosphate buffer was prepared by mixing approximately 50:50 (v/v) of 1 M di-potassium hydrogen phosphate (J.T. Baker, Deventer, the Netherlands) and 1 M potassium dihydrogen phosphate. At the time of usage, whey permeate and yeast extract parts were mixed in a sterile flasks in a 85:15 ratio.

Cereal Matrices

Two kinds of cereal matrices were prepared for the vitamin B_{12} production: (1) barley malt matrix (BMM; 33% w/v barley malt) and (2) barley flour matrix (BFM; 6% w/v wholemeal barley flour). The cereal matrices were prepared by boiling 33% w/v barley malt (Laihian Mallas, Laihia, Finland) and 6% w/v wholemeal barley flour (Myllyn Paras, Hyvinkää, Finland) for 2 min. Both cereal matrices were then autoclaved and transferred to sterile falcon tubes for fermentation.

3.2.3 Culture Preparation

Frozen cultures were thawed, inoculated on agar plate and incubated anaerobically at 30°C for 3-4 days. Three individual colonies were transferred to three falcon tubes, each filled with 5 mL of SWP medium (three biological replicates) and incubated anaerobically at 30°C for each strain. The strains were then subcultured (2% v/v) 3 times in triplicates for 3-4 days at 30°C in 5 mL of SWP medium. The incubation took place in anaerobic jars equipped with Anaerocult® A.

3.2.4 Vitamin B_{12} Production in SWP Medium

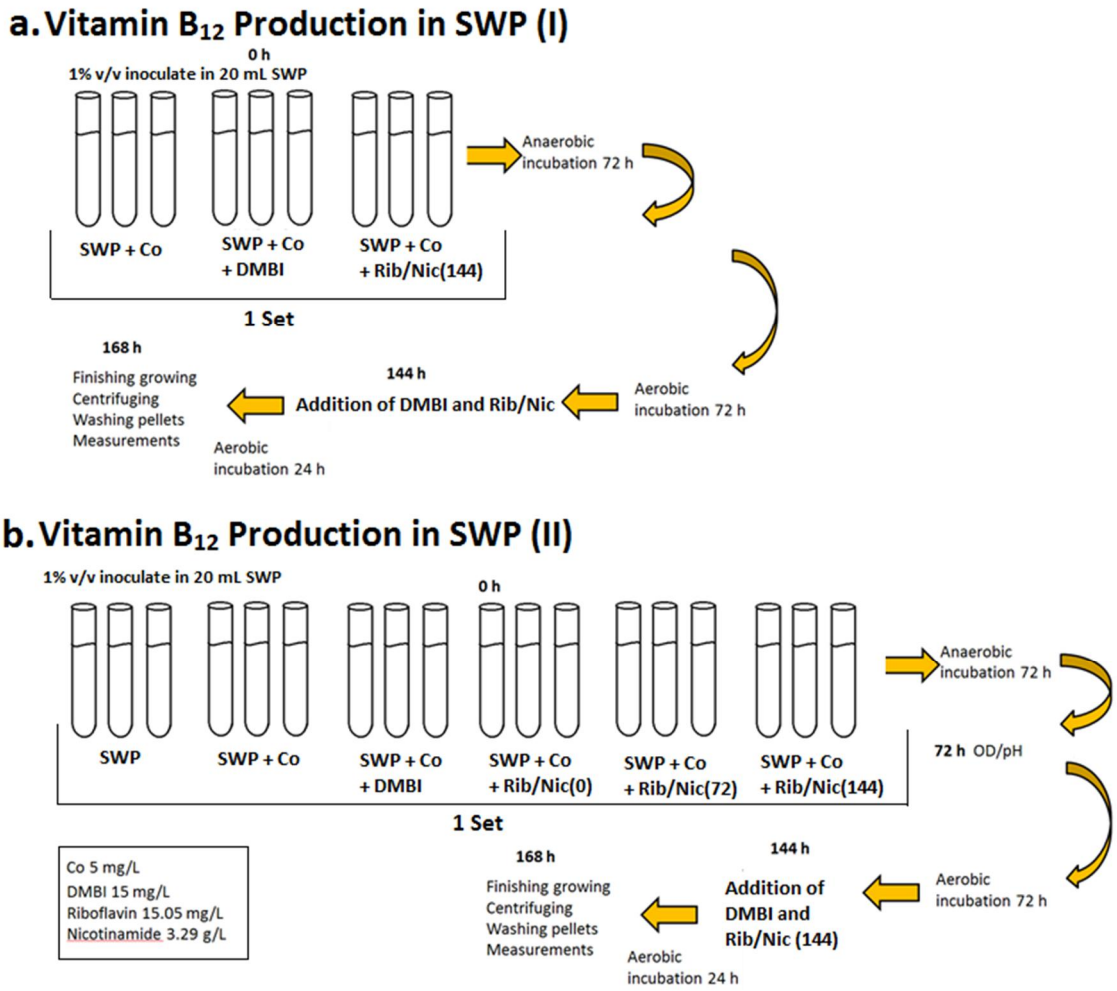

Figure 10. The flowchart of the 168-hour-vitamin B_{12} production in the SWP medium **a.** Vitamin B_{12} production in the SWP medium based on the type of precursors and **b.** Vitamin B_{12} production in the SWP medium based on the timing of precursors addition.

SWP I:Vitamin B_{12} Production with Addition of Precursors

The 168-hour-fermentations were carried out in triplicate in 50 mL sterile falcon tubes (CELLSTAR®; Greiner Bio-one). The three subcultured strains were inoculated (1% v/v) in 20 mL of fresh SWP. Based on the type of precursors, three conditions were tested in SWP medium (Figure 10a): (1) with cobalt (II) chloride (Sigma-Aldrich Co., UK); (2) with cobalt (II) chloride and DMBI; and (3) with cobalt (II) chloride, riboflavin and nicotinamide (Sigma-Aldrich Co., China). The amount of cobalt (II) chloride, DMBI, riboflavin and nicotinamide were 5 mg/L, 15 mg/L, 15.05 mg/L and 3.29 g/L, respectively, as recommended by Hoerig and Renz (1980) and Hugenschmidt et al. (2010). Cobalt (II) chloride was added at 0 h while the rest of precursors were added at 144 h. Before addition, all precursors were made in MQ-water and sterile filtered (0.2 µm filter; Acrodisc® 32 mm Syringe Filters; Pall Corporation, Cornwall, UK).

All strains were incubated in anaerobic jars equipped with Anaerocult® A at 30°C for 72 h, followed by 96 h aerobic incubation. To obtain aerobic conditions, the tubes were slightly opened under sterile conditions, then closed again and incubated on a shaking plate (150 rpm, CERTOMAT® MO II; Sartorius AG) maintained at 30°C. After the addition of precursors at 144 h, the samples were further incubated aerobically for 24 h before harvesting vitamin B_{12}. Sample aliquots were collected at 0, 72, and 168 h for pH, optical density, sugar and acid analyses. At 72 h, SWP medium sampling was done by taking 1.9-2.0 mL in Eppendorf tubes. After measuring the OD and pH, the tubes were centrifuged (12000 rpm, 10 min at 4°C; himac CT15RE, Hitachi Koki Co., Ltd., Japan). The supernatants were filtered through 0.2 µm filter and stored at -20°C for sugar and acid analyses. At the end of incubation, the strains grown in SWP medium were centrifuged (12000 rpm, 15 min at RT; Centrifuge 5810R, Eppendorf AG, Germany). The supernatants were collected in 15 mL tubes at -20°C for sugar and acid analyses. The cell pellets were washed with 10 mL phosphate buffered saline (PBS) (Dulbecco A; OXOID Ltd., England) and centrifuged again. The second supernatants were discarded and the pellets were stored at -20°C for vitamin B_{12} analysis.

SWP II : Timing of Riboflavin/Nicotinamide Addition

Two strains of *P. freudenreichii* (1 and 3) were tested. In this experiment the effect of precursor addition on vitamin B_{12} by the tested strains were evaluated. Similarly to the SWP I experiment, the strains were first inoculated (1% v/v) in 20 mL fresh SWP in 50 mL sterile falcon tubes. However, six treatments were carried out based on the type of precursors and the time of adding the precursors (Figure 10b): (1) non-supplemented SWP; (2) with cobalt (II) chloride; (3) with cobalt (II) chloride and DMBI; (4) with cobalt (II) chloride, riboflavin and nicotinamide (rib/nic; 0 h); (5) with cobalt (II) chloride and rib/nic (72 h); and (6) with cobalt (II) chloride and rib/nic (144 h). All precursors had the same concentration as in the SWP I experiment. Cobalt (II) chloride was added at 0 h and DMBI was added at 144 h. All samples were then incubated in similar condition as in the SWP I experiment. Sample aliquots were collected at 0, 72, and 168 h for pH, optical density, sugar and organic acid analyses, followed by harvesting the cells for vitamin B_{12}.

One more thing that was different in the SWP II experiment was the preparation of riboflavin and nicotinamide solutions. In the first experiment, riboflavin and nicotinamide were prepared separately. However, riboflavin has poor solubility in water, making it difficult to dissolve. Frost (1946) reported that at pH 5.0 5% nicotinamide increased the solubility of riboflavin by nine times. Hence, different concentrations of riboflavin: 0.05%, 0.1%, 0.17%, 0.25% and 0.5% were diluted with 3.29 g nicotinamide in 10 mL water (Figure 11). The appropriate solution with the required concentration of precursors was then used in the vitamin B_{12} production.

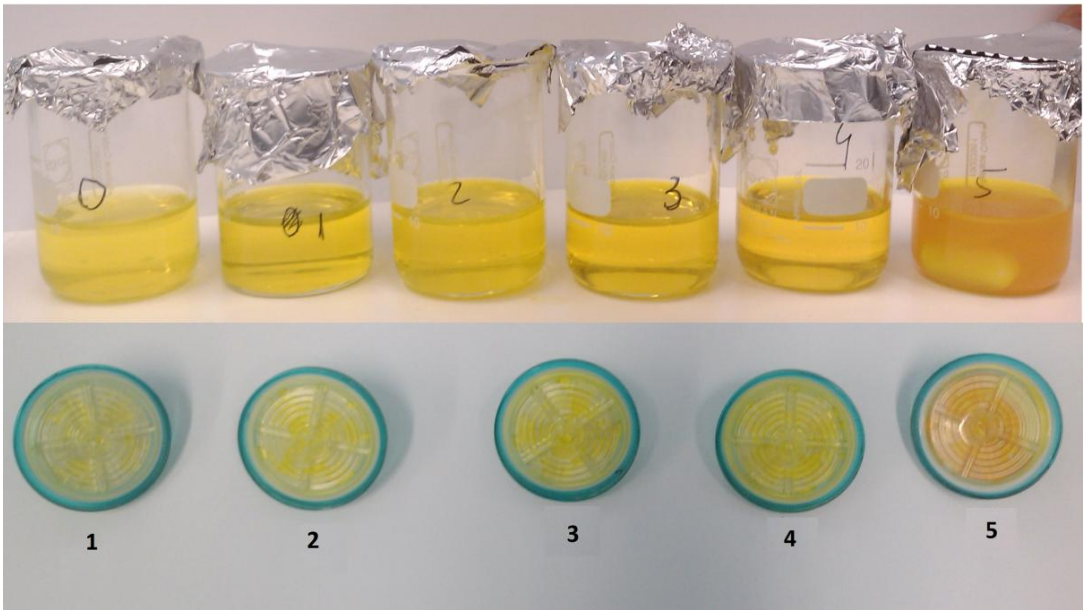

Figure 11. The solubility test of riboflavin and nicotinamide. Concentration of riboflavin: (1) 0.05%; (2) 0.1%; (3) 0.17%; (4) 0.25%; and (5) 0.5%. The upper picture shows the opaqueness of the solution increased as higher concentration of riboflavin was added. The bottom picture shows the insoluble riboflavin trapped in the filter. Solution 3 was chosen in the vitamin B_{12} production.

3.2.5 Vitamin B_{12} Production in Cereal Matrices

The 168-hour-fermentations were carried out in triplicate in 50 mL sterile falcon tubes. The three subcultured strains were inoculated (1% v/v) in 20 mL fresh barley malt and barley flour matrices. Based on the type of precursors, three conditions were tested in cereal matrices (Figure 12): (1) non-supplemented cereal matrices; (2) with DMBI; and (3) with cobalt (II) chloride and DMBI. The amount of cobalt (II) chloride and DMBI were 5 mg/L and 15 mg/L, respectively. Cobalt (II) chloride was added at 0 h while DMBI was added at 144 h. All samples were then incubated in similar condition as in the SWP experiment. The bacterial growth in cereal matrices was carried out by measuring viable cell counts (CFU/g).

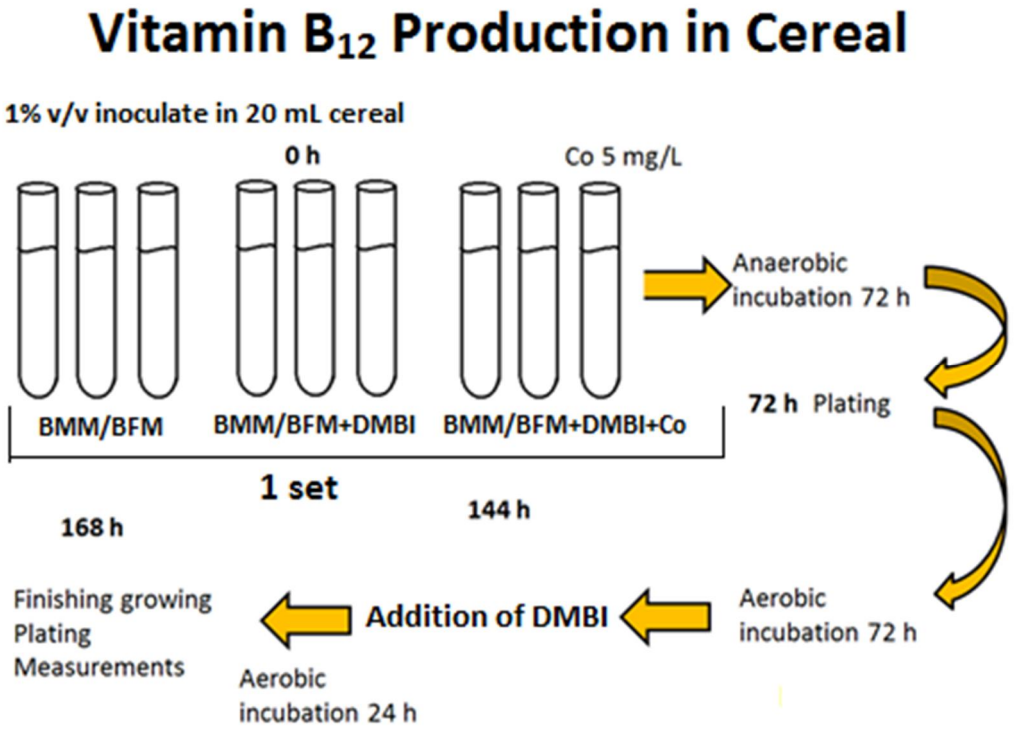

Figure 12. Flowchart of 168-hour-vitamin B_{12} production in cereal matrices; barley malt matrix (BMM; 33% w/v barley malt) and barley flour matrix (BFM; 6% w/v wholemeal barley flour).

3.2.6 Growth Measurements

Optical density. The optical density (OD) was measured at 600 nm using a spectrophotometer (Novaspec II, Amersham Pharmacia Biotech). The culture was diluted 20-fold and measured against fresh SWP medium.

pH. At 72 h, the pH of culture samples was measured with pH-papers in the range of 3.6-6.1 (pH-Fix; Macherey-Nagel GmbH, Germany). The pH of 168 h culture samples was measured with pH meter (PHM220 MeterLab™; Radiometer Analytical SAS, France). The pH of the cereal matrices was measured at 0 h and 168 h.

Cell enumeration. About 1 g of fermented cereal samples were serially diluted in PBS buffer and 100 µL of appropriate dilutions was plated by surface spreading method on agar plates for viable cell counts. The plates were incubated anaerobically at 30°C for 5-6 days. Cell counts were performed and mean values were presented.

3.2.7 Vitamin B_{12} Analysis

Vitamin B_{12} is subject to light degradation. Thus, extraction, sample enrichment, MBA and UHPLC analyses were conducted under subdued light.

Preparation of Cyanocobalamin Standard Solution

The preparation of standard solution was adapted and modified from Indyk et al. (2002). 4 mg of cyanocobalamin (Supelco, Bellefonte, PA, USA) was accurately weighed into a 20 mL flask and diluted to volume with 25% ethanol (Altia Oyj, Rajamäki, Finland), called as the stock solution I (0.2 mg/mL). To determine the actual concentration of the stock solution I, the solution was diluted 10-fold in 25% ethanol and its absorbance was measured against 25% ethanol at 361 nm using spectrophotometer (Lambda 25 UV/Vis; Perkin Elmer Inc., USA). The result was calculated using molar absorption coefficient ($E_{361}=28.1*10^3$). The stock solution I was stable for 3 months at 4°C.

At the day of analysis, the calibrant was prepared by diluting the stock solution I 100-fold, called as the stock solution II (2 µg/mL). The stock solution II was further diluted 1:200, then 1:100 (0.1 ng/mL) with 6.2 pH extraction buffer.

Vitamin B_{12} Extraction

SWP medium. The procedure for extracting vitamin B_{12} was adapted and modified from Kelleher and Broin (1991). About 0.1 g of thawed cell pellets was weighed into a 30 mL-centrifuge tube and suspended in 10 mL of buffer (pH 4.5) containing 8.3 mmol/L sodium hydroxide and 20.7 mmol/L glacial acetic acid (Riedel-de Haën GmbH, Seelze, Germany). 100 µL of 1% sodium cyanide (Sigma-Aldrich Chemie, Steinheim, Germany) was added into the tube, followed by heating the suspension for 30 min. in 100°C boiling water. The addition of cyanide was meant to convert any forms of vitamin B_{12} to cyanocobalamin. After cooling in ice bath, the tubes were then centrifuged (8000 rpm, 10 min at RT; Z 323, Hermle, Germany) and supernatants were collected in another 30 mL tube. The pellets were re-suspended with 5 mL of buffer (pH 6.2, adjusted with 10% sodium hydroxide) and centrifuged again. The second supernatants were combined with the first supernatants. The pH of supernatants was adjusted to 6.2 by adding 3% sodium hydroxide. The extracts were then filtered (90 mm qualitative filter paper; VWR International, Belgium)

into a 25 mL flask and diluted to volume with pH 6.2 buffer. The extracts were ready for vitamin analysis by MBA or UHPLC or stored at -20°C. Using the same procedure as explained above, a blank sample and certified reference material (pig liver; BCR 487) were also extracted. The pig liver was used as the reference to evaluate the accuracy and precision of the quantification procedures.

Cereal matrices. About 1-2 g of fermented cereal matrices was extracted with 10 mL of buffer (pH 4.5). To break down the starch, 1 mL of α-amylase (50 mg/mL, produced from *Aspergillus oryzae*; Sigma-Aldrich Chemie, Steinheim, Germany) was added together with 100 µL of 1% sodium cyanide. The addition of amylase helped to ease filtration of the extracts for MBA and immunoaffinity purification. Prior to boiling, the tubes were incubated for 40 min at 37°C in a water bath equipped with a shaking plate. The tubes were then centrifuged, filtered and stored as described in SWP medium extraction.

Sample Enrichment by Immunoaffinity Column

Prior to UHPLC analyses, vitamin B_{12} extracts from the cereal matrices were further purified and concentrated through "EASI-EXTRACT® Vitamin B_{12}" (R-Biopharm Rhone Ltd, Glasgow, Scotland), an immunoaffinity column containing monoclonal antibody with high affinity to cyanocobalamin. The procedures were adapted and modified from Campos-Giménez et al. (2008), Marley et al. (2009) and Guggisberg et al. (2012). The thawed extracts were first filtered through 0.45 µm syringe filter (Acrodisc® 32 mm; Pall Corporation, Cornwall, UK). The sample extracts equivalent to the level of vitamin B_{12} above the quantitation limit (LOQ) of UHPLC was then purified by immunoaffinity column. The sample extracts loaded into the column should not exceed the saturation of the immunoaffinity column (1.0 µg of vitamin B_{12}).

Firstly, the immunoaffinity columns were conditioned to room temperature. The storage buffer was drained by gravity. Appropriate volume of extracts (1-15 mL) was passed through the columns followed by washing with 10 mL of Milli-Q water (Q-POD® Element; Merck Millipore). The columns were drained by inserting about 50 mL of air using a syringe. The vitamin B_{12} absorbed in the column matrix was eluted with 3 mL of methanol and rinsed with additional 0.5 mL of methanol. 20 mL of air was injected to remove any methanol from the columns. On a heating plate (50-60°C), the eluates were evaporated to dryness under a stream of nitrogen.

The samples were reconstituted in 300 µL of Milli-Q water. The extracts were then transferred to UHPLC vials (Waters Corporation, USA) and analysed with UHPLC/UV.

Analysis by Microbiological Assay (MBA)

MBA protocols were adapted and modified from established reference procedures (Kelleher and Broin, 1991). The assay medium was first prepared by dissolving 3.1 g of vitamin B_{12} assay medium and 75 µL of Tween 80 in 50 mL of Milli-Q water, followed by boiling for 2 min. After cooling, the pH was adjusted to 6.2 with 10% NaOH. The medium was filtered into a sterile glass bottle. The filtered medium was kept on ice until inoculation with *Lactobacillus delbrueckii* (ATCC 7830). The MBA analysis was performed in 96-well microtiter plates (Corning Inc., New York, USA). A standard curve was prepared in quadruplicate, comprising eight cyanocobalamin concentrations from 0-8 pg (0-80 µL)/well. Extraction buffer (20-100 µL) was then pipetted into these wells so that each well contained 100 µL. Based on an estimation of vitamin B_{12} content (20-50 pg/mL), two dilutions of each sample extract were prepared in 10 mL flasks with pH 6.2 extraction buffer. 100 µL of vitamin B_{12} extracts was pipetted to the remaining wells. A vial of glycerol-cryopreserved *L. delbrueckii* was rapidly thawed. 100 µL of *L. delbrueckii* culture was then added to the cooled 50 mL of assay medium. 200 µL of inoculated medium was added to every well using an eight-channel pipette. Each plate was covered with a plate sealer and incubated for 19 hours at 35°C. The OD of the wells was then measured at 595 nm on a microplate reader (Multiskan EX; Labsystems, Finland). Vitamin B_{12} concentrations were calculated according to the calibration curve and expressed in µg/mL and ng/g for vitamin B_{12} production in the SWP medium and the cereal matrices, respectively.

Analysis by Ultra High-Performance Liquid Chromatography (UHPLC)

Vitamin B_{12} was also quantified with a newly developed UHPLC method (Chamlagain et al. submitted). The separation was performed on a Waters Acquity UHPLC system (Milford, Massachusetts, USA) using a 2.1 X 100 mm High Strength Silica (HSS) T3 1.8 µm column. The elution of cyanocobalamin was detected at 361 nm on a Waters photodiode array detector. The method used two mobile phases where solution A was 0.025% (v/v) trifluoroacetic acid (TFA) in

Milli-Q water and solution B was 0.025% TFA in acetonitrile. All solvents were previously filtered through 0.2 µm hydrophilic polypropylene membrane filters (Pall Corporation, Michigan, USA). Six concentrations of cyanocobalamin (0.016-0.8 ng/µL) were prepared from the stock solution I for a standard curve. The sample injection volume was 5-15 µL (vitamin B_{12} extracts) and 15 µL (cyanocobalamin standards). A flow rate of 0.320 mL/min was maintained throughout the duration of each run (10 min). Elution was done in the following conditions: 0-0.50 min, 95% A and 5% B were combined in a linear gradient; 0.50-5.00 min, 95% A and 5% B were combined in a linear gradient to a final proportion of 60% A and 40% B; 5.00-6.00 min, 60% A and 40% B were maintained; 6.00-7.00 min, 60% A and 40% B were combined in a linear gradient to a final proportion of 95% A and 5% B, which were maintained until 10.00 min. The final results were expressed in µg/mL and ng/g for vitamin B_{12} production in SWP medium and cereal matrices, respectively.

3.3 Results

According to the media (SWP and cereal matrices) utilized to produce vitamin B_{12}, the results are divided into three parts: (1) vitamin B_{12} production in DMBI and riboflavin/nicotinamide added SWP (SWP I); (2) vitamin B_{12} production with different timing of riboflavin/nicotinamide addition (SWP II) and (3) vitamin B_{12} production in the cereal matrices. In each part, the results of growth measurements (pH, OD and cell counts) are presented, followed by vitamin B_{12} concentrations.

3.3.1 Vitamin B_{12} Production in DMBI and Riboflavin/Nicotinamide Added SWP

3.3.1.1 Growth of *Propionibacterium* Strains

pH

The pH of the SWP medium fermented by the *P. freudenreichii* strains during the 168-hour-vitamin B_{12} production is shown in Figure 13. Within 72 hours of anaerobic fermentation, a decrease from pH 6.5 to around pH 5.0-5.3, depending on the strains, was observed. After 96 hours of aerobic fermentation, the pH of the SWP medium further reduced to pH 4.9-5.0 for all three strains.

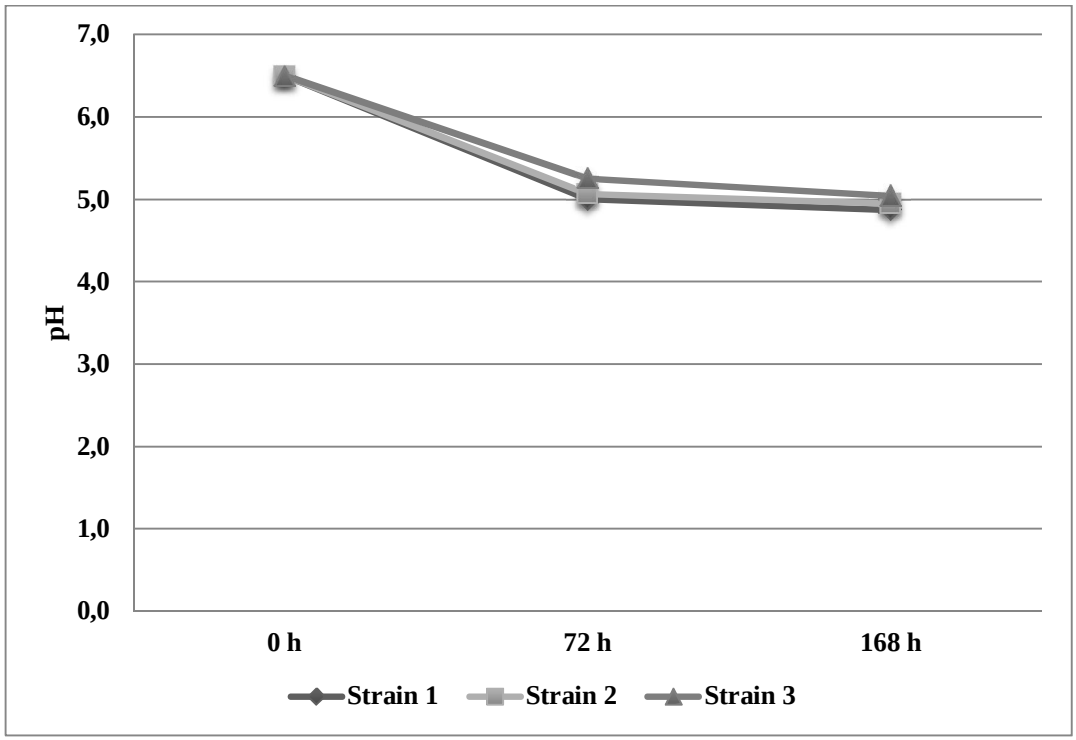

Figure 13. The pH in of SWP medium during the 168-hour-vitamin B_{12} production by the *P. freudenreichii* strains 1, 2 and 3.

Optical Density

The bacterial growth in the SWP I experiment is presented in Figure 14. At 72 h, the strain 1 showed the highest biomass growth with the average OD of 11.0. Approximately half OD compared to that in the strain 1 was observed in the strain 2 and 3 with the average of 5.9 and 5.8, respectively. However, following 96 hours of aerobic fermentation, higher increase of OD was observed in the SWP medium fermented by the strain 2 and strain 3, giving the average of 7.9 and 7.8, respectively. While at 168 h, the strain 1 showed insignificant increase of OD (P=0.166), giving the final OD of 11.6.

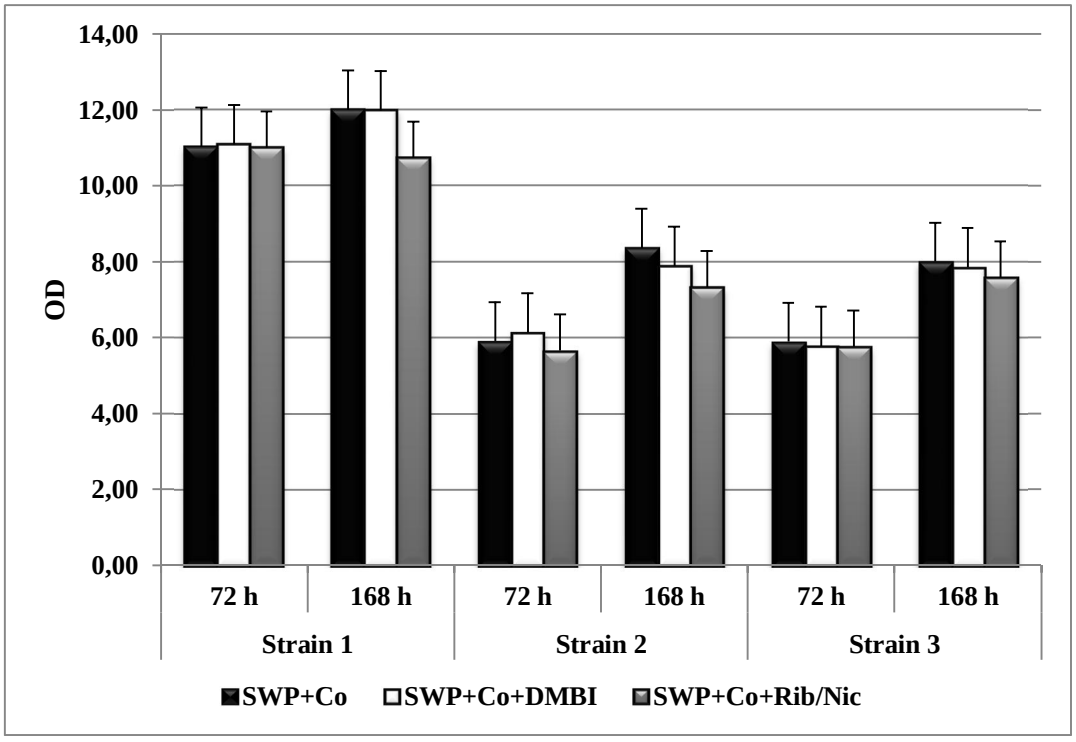

Figure 14. The optical density of the *P. freudenreichii* strains 1, 2 and 3 in the SWP medium with the addition of cobalt (II) chloride, DMBI, and riboflavin/nicotinamide during the 168-hour-vitamin B_{12} production.

3.3.1.2 Vitamin B_{12} Content

Effect of DMBI Supplementation

The effect of various precursors on the level of vitamin B_{12} produced by the three *P. freudenreichii* strains is shown in Figure 15. The addition of 15 mg/L DMBI increased vitamin B_{12} content by almost 2-fold to 1.7 µg/mL in the SWP medium

inoculated with the strain 1. An increase of vitamin B_{12} content due to the addition of DMBI was also observed in the medium inoculated with the strain 2, yielding up to 3-fold rise to 1.9 µg/mL. On the contrary, compared to the vitamin B_{12} content produced in the cobalt-added SWP medium, the vitamin B_{12} production did not increase with the addition of DMBI in the SWP medium inoculated with the strain 3.

Effect of Riboflavin/Nicotinamide Supplementation

The addition of 15.05 mg/L of riboflavin and 3.29 g/L of nicotinamide did not increase vitamin B_{12} production in the SWP medium fermented by the strain 1. In the SWP medium fermented by the strain 2, additional supplementation of riboflavin/nicotinamide led to a similar (P=0.358) level of vitamin B_{12} as vitamin B_{12} content produced in SWP medium supplemented by only cobalt (II) chloride. Strain 3 was the only strain showing an increase in vitamin B_{12} concentration with the riboflavin/nicotinamide supplementation on day 6 (144 h), yielding 1.1 µg/mL of vitamin B_{12}.

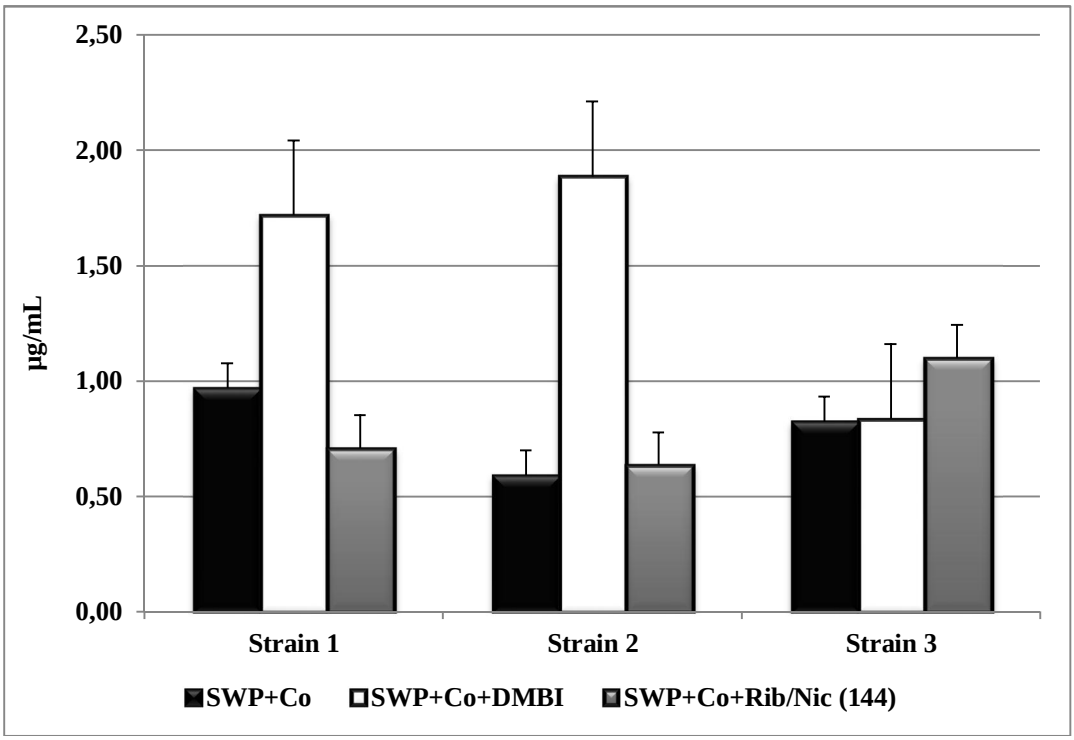

Figure 15. The vitamin B_{12} concentration produced by the *P. freudenreichii* strains 1, 2 and 3 in the SWP medium supplemented with cobalt (II) chloride, DMBI, and riboflavin/nicotinamide. Cobalt (II) chloride was added at 0 h, while DMBI and riboflavin/nicotinamide were added at 144 h. Vitamin B_{12} was quantified by UHPLC (means and standard deviations of 3 biological replicates).

3.3.1.3 Analysis of Vitamin B_{12} by MBA and UHPLC

Table 7 shows the vitamin B_{12} concentrations produced in the SWP I medium by the *P. freudenreichii* strains, analysed by the MBA and the UHPLC method. The MBA-determined vitamin B_{12} concentrations were up to 34% higher than the UHPLC-measured vitamin B_{12} concentrations. The ratio of the vitamin B_{12} levels obtained by the two methods differed depending on the *P. freudenreichii* strains. Both methods showed similar vitamin B_{12} levels for the samples fermented by the strain 3, with average discrepancy of 9.7%. While bigger discrepancy between the two methods was observed in the samples fermented by the strain 1 and 2, giving a difference of 20.5% and 28.9%, respectively.

Table 7. The vitamin B_{12} concentrations produced by the three *P. freudenreichii* strains in the SWP medium, analysed by microbiological assay and the UHPLC method.

Conditions	Strains								
	1			2			3		
	Vit. B_{12} conc. (µg/mL)			Vit. B_{12} conc. (µg/mL)			Vit. B_{12} conc. (µg/mL)		
	MBA	UHPLC	MBA/UHPLC	MBA	UHPLC	MBA/UHPLC	MBA	UHPLC	MBA/UHPLC
SWP+Co	1.20±0.26[a]	0.97±0.07[b]	1.24	0.76±0.13[a]	0.59±0.09[a]	1.28	0.84±0.03[a]	0.82±0.03[a]	1.02
SWP+Co+DMBI	2.03±0.02[a]	1.72±0.04[a]	1.18	2.34±0.11[a]	1.89±0.06[a]	1.24	0.88±0.03[a]	0.83±0.03[a]	1.05
SWP+Co+Rib/Nic(144)	0.84±0.02[b]	0.71±0.03[a]	1.19	0.85±0.13[b]	0.63±0.06[b]	1.34	1.33±0.07[a]	1.10±0.08[a]	1.21

[a] All values obtained represent mean ± SEM (n = 3)
[b] All values obtained represent mean ± SEM (n = 2)

3.3.2 Vitamin B_{12} Production with Different Timing of Riboflavin/Nicotinamide Addition

3.3.2.1 Growth of *Propionibacterium* Strains

pH

The changes in the pH of the SWP II experiment (Figure 16) showed similar trend to the SWP I experiment (Figure 13). After 3 days of anaerobic fermentation, the initial pH 6.5 decreased to pH 5.0 and 5.2 for the strain 1 and 3, respectively. Further reduction of pH during the 4 days of aerobic fermentation was also observed in the strain 1 and 3, giving the final values of pH 4.8 and 5.0, respectively.

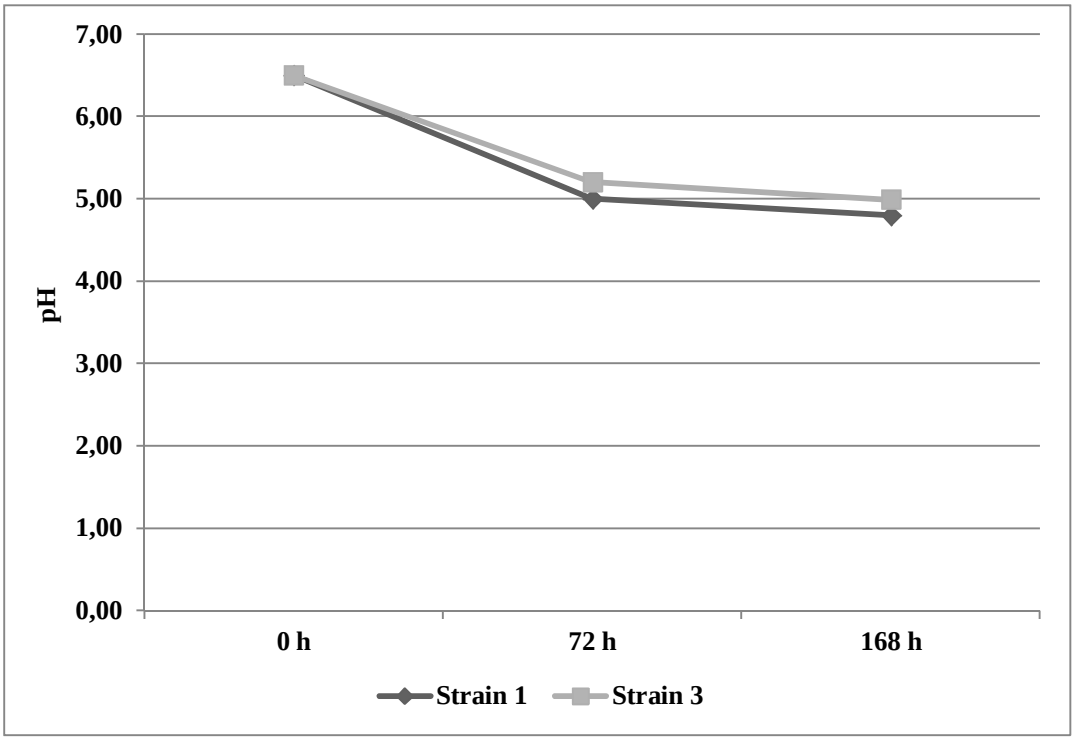

Figure 16. The pH in the SWP medium during the 168-hour-vitamin B_{12} production by the *P. freudenreichii* strains 1, 2 and 3.

Optical Density

The changes in the OD in the SWP II experiment (Figure 17) showed similar trends to the OD in the SWP I experiment (Figure 14), particularly in the samples treated with the same conditions. Approximately twice higher OD in the strain 1 than in the strain 3 was observed in all six conditions after 72 h of anaerobic fermentation. At the end of fermentation, most of the samples showed further

biomass growth, in which the increase of the OD in the strain 3 (P=0.001) was significantly higher than that in the strain 1 (P=0.210). Furthermore, the timing of adding riboflavin/nicotinamide seemed to influence the growth of the *P. freudenreichii* strains, particularly for the strain 3. When riboflavin/nicotinamide was added at 0 and 72 h, the OD of the strain 3 increased from 6.9 to 9.5 and from 6.7 to 9.4, while a slight increase of the OD from 7.4 to 7.8 was observed when riboflavin/nicotinamide was added at 144 h.

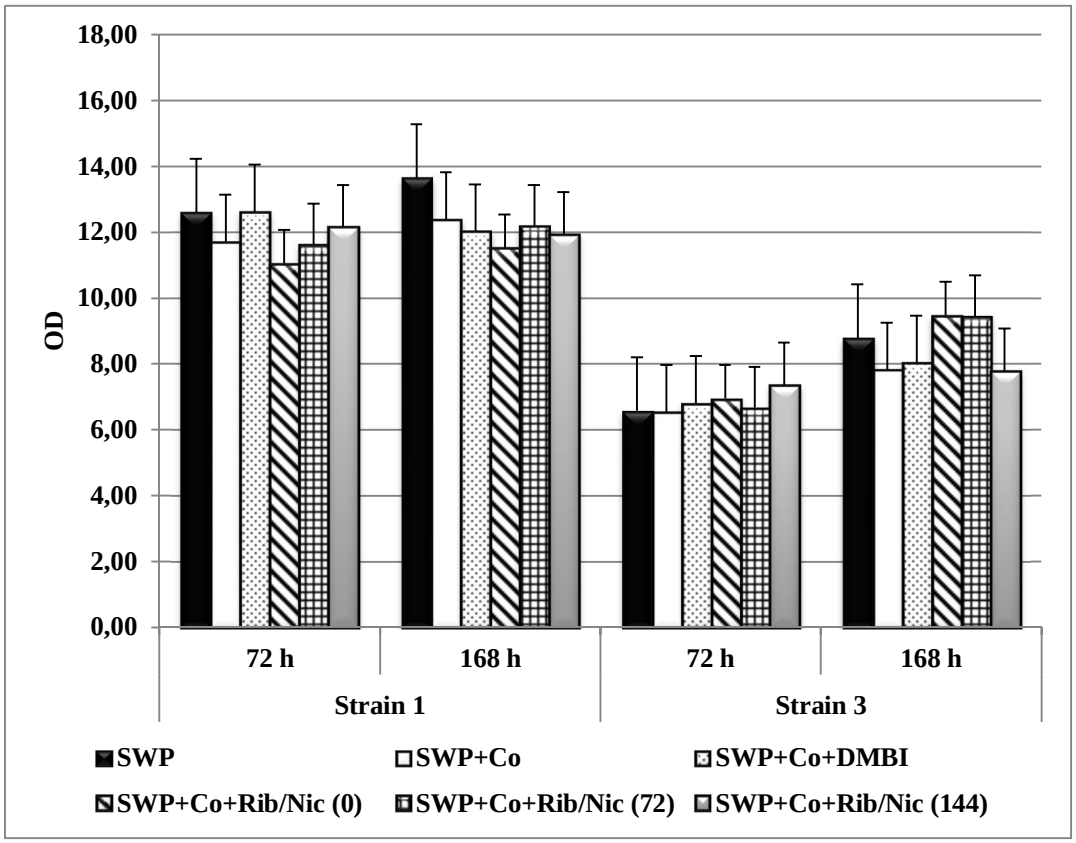

Figure 17. The optical density of the *P. freudenreichii* strains 1 and 3 in the SWP medium, treated with six conditions during the 168-hour-vitamin B_{12} production. Riboflavin and nicotinamide were added at different time points; 0 h, 72 h and 144 h.

3.3.2.2 Vitamin B_{12} Content

Since cobalt is the central atom of vitamin the B_{12} structure, the effect of cobalt (II) chloride addition on vitamin B_{12} production was investigated by fermenting SWP medium with *P. freudenreichii* strains without the addition of cobalt (II) chloride or any other precursors. The effect of different timing in adding riboflavin/nicotinamide on vitamin B_{12} conentrations was also investigated.

Effect of Cobalt Supplementation

The effect of cobalt addition on vitamin B_{12} concentrations was quite evident. Without the addition of any precursors, the strain 1 and 3 produced lowest yields of vitamin B_{12} (Figure 18). With the supplementation of cobalt (II) chloride, an increase of vitamin B_{12} content up to 4-fold was observed in the strain 1 and 3, yielding 0.9 and 0.7 µg/mL of vitamin B_{12}, respectively.

Effect of DMBI Supplementation

Compared to the vitamin B_{12} contents produced in the SWP I experiment under the same condition (Figure 15), additional supplementation of DMBI in the cobalt-added SWP medium showed similar vitamin B_{12} concentrations (Figure 18), yielding 1.7 and 0.8 µg/mL for the strains 1 and 3, respectively. As expected, insignificant increase (P=0.065) due to the addition of DMBI was observed in the samples fermented by the strain 3.

Effect of Timing of Riboflavin/Nicotinamide Supplementation

The supplementation of riboflavin/nicotinamide enhanced vitamin B_{12} production only in the samples inoculated with the strain 3 (Figure 18), which was also observed in the SWP I experiment (Figure 15). Compared to the vitamin B_{12} contents produced in the unsupplemented SWP medium, approximately 4-9 fold rise of vitamin B_{12} amount was produced by the strain 3, depending on the time of addition of riboflavin/nicotinamide. The timing of adding riboflavin/nicotinamide influenced the amount of vitamin B_{12}; the strain 1 and 3 synthesized higher vitamin B_{12} content when riboflavin/nicotinamide was added at the beginning of fermentation than at the latter stage. However, more significant increase of vitamin B_{12} due to the different timing in adding the precursors was observed in the strain 3 (P=0.006) than in the strain 1 (P=0.028). In the samples inoculated with the strain 3, the addition of riboflavin/nicotinamide at 0 h and 72 h produced 2-fold vitamin B_{12} concentrations than that produced when the precursors were added at 144 h, yielding 2.2, 2.1 and 1.1 µg/mL of vitamin B_{12}, respectively. Particularly for the strain 3, higher vitamin B_{12} concentrations due to the early addition of riboflavin/nicotinamide were also supported by higher OD after 168 hours of fermentation (Figure 17).

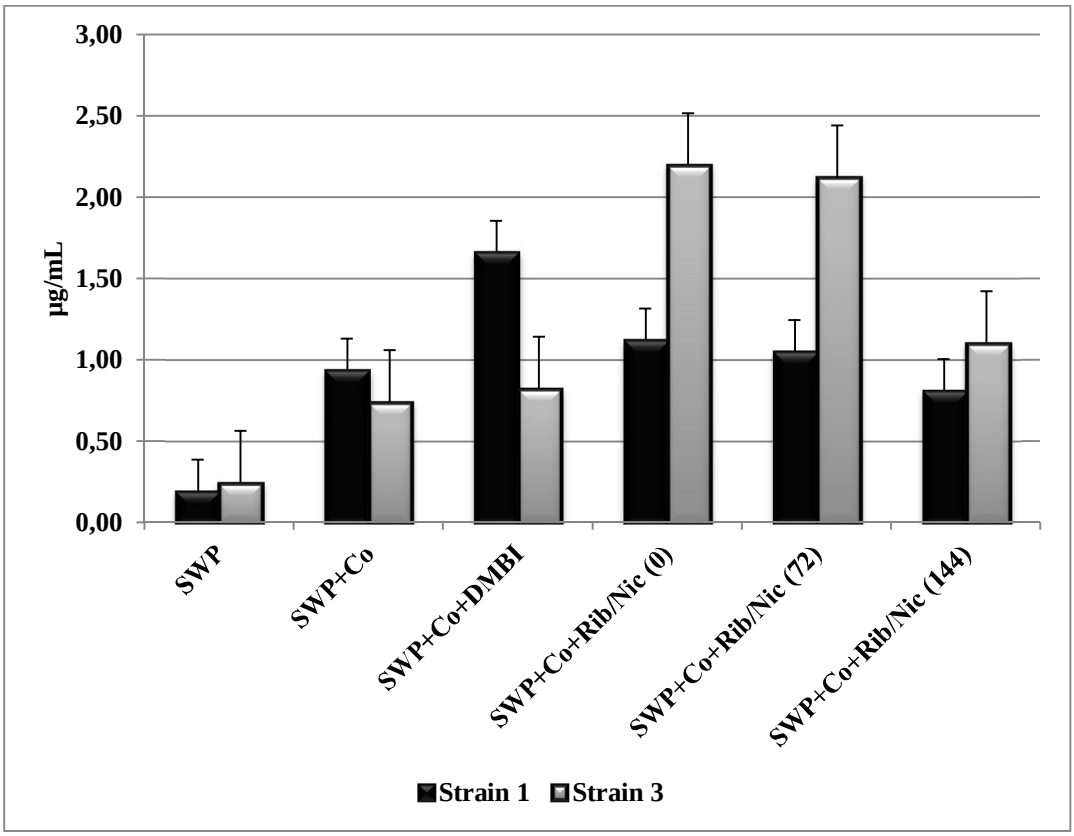

Figure 18. The vitamin B_{12} concentrations produced by the *P. freudenreichii* strain 1 and 3 in the SWP medium, treated with six conditions during the 168-hour-vitamin B_{12} production. Riboflavin and nicotinamide were added at different time points; 0 h, 72 h and 144 h. Vitamin B_{12} was quantified by UHPLC (means and standard deviations of 3 biological replicates).

3.3.2.3 Analysis of Vitamin B_{12} by MBA and UHPLC

Table 8 shows the vitamin B_{12} concentrations produced in the SWP medium under six different conditions, analysed by MBA and UHPLC. The MBA/UHPLC ratio in the SWP II experiment showed similar trends to the SWP I experiment; all MBA-determined vitamin B_{12} concentrations were higher than vitamin B_{12} levels measured by the UHPLC method. However, compared to the MBA/UHPLC ratio in the SWP I experiment, closer discrepancy was achieved, giving difference between 2-20%.

Table 8. The vitamin B_{12} concentrations produced by the two *P. freudenreichii* strains in the SWP medium, analysed by microbiological assay and UHPLC method.

Conditions	Strains					
	1			3		
	Vit. B_{12} conc. (µg/mL)			Vit. B_{12} conc. (µg/mL)		
	MBA	UHPLC	MBA/ UHPLC	MBA	UHPLC	MBA/ UHPLC
SWP	0.21±0.01[a]	0.19±0.01[a]	1.07	0.25±0.01[a]	0.24±0.01[a]	1.02
SWP+Co	1.11±0.02[a]	0.93±0.03[a]	1.19	0.86±0.01[a]	0.74±0.03[a]	1.17
SWP+Co+DMBI	1.78±0.01[a]	1.66±0.01[a]	1.07	0.91±0.03[a]	0.82±0.02[a]	1.11
SWP+Co+Rib/Nic(0)	1.28±0.05[a]	1.12±0.08[a]	1.14	2.34±0.18[a]	2.20±0.10[a]	1.07
SWP+Co+Rib/Nic(72)	1.13±0.04[a]	1.05±0.07[a]	1.08	2.20±0.07[a]	2.12±0.07[a]	1.04
SWP+Co+Rib/Nic(144)	0.88±0.03[a]	0.81±0.02[a]	1.09	1.18±0.04[a]	1.10±0.17[a]	1.07

[a] All values obtained represent mean ± SEM (n = 3)

3.3.3 Vitamin B_{12} Production in Cereal Matrices

3.3.3.1 Growth of *Propionibacterium* Strains

pH

The change of the pH during the 168-hour-fermentation in the BMM and BFM is shown in Figure 19. At the end of fermentation, the pH of the BMM samples fermented by the strains 1, 2 and 3 decreased from pH 5.6 to pH 4.6, 4.5 and 4.5, respectively. However, fermentation did not markedly reduce the pH in the barley BFM samples; the pH at 168 h remained close to the initial pH of the matrix (Figure 19).

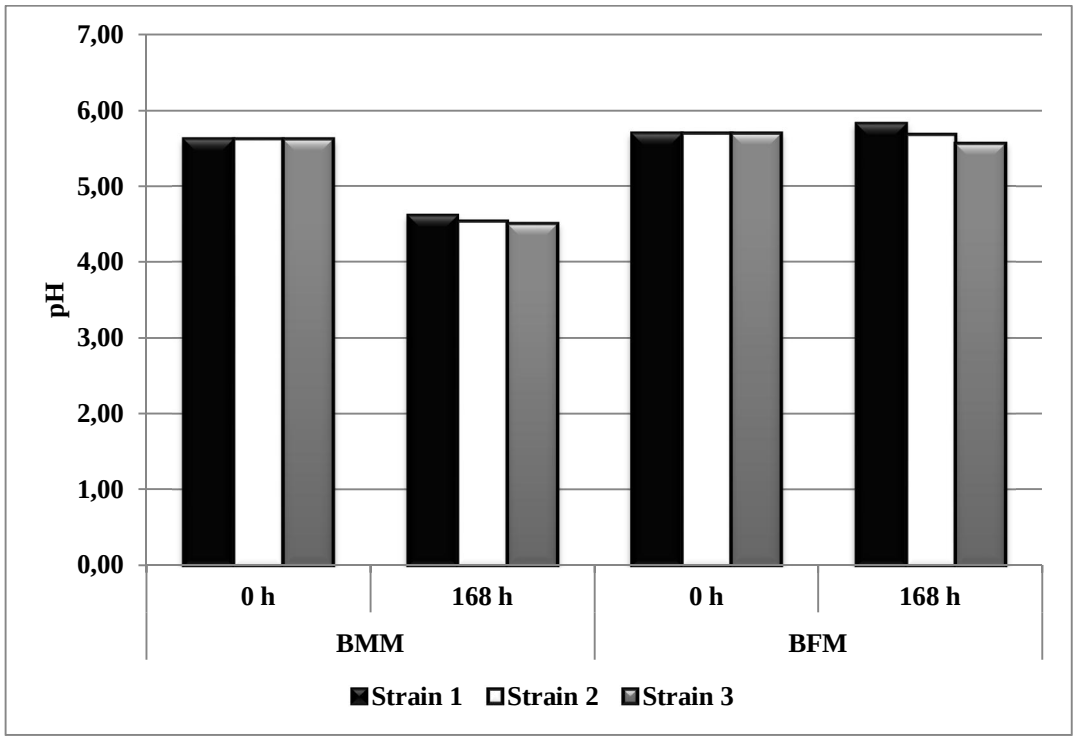

Figure 19. The pH in the cereal matrices during the 168-hour-B_{12} production by the *P.freudenreichii* strains 1, 2 and 3. BMM: 33% w/v barley malt and BFM: 6% w/v wholemeal barley flour.

Cell Counts

At the beginning of vitamin B_{12} production, the cell counts in both matrices were at the same level (Figure 20), with average of 8.0 and 8.1 log CFU/g for *P. freudenreichii* strains grown in the BMM and the BFM, respectively. After 72 hours of anaerobic fermentation, the bacterial population of *P. freudenreichii* strains in the fermented BMM increased up to 60, 35 and 120-fold for the strain 1, 2 and 3. While at 72 h, a log factor increase of cell counts was observed in the fermented BFM. The cell counts in both matrices remained the same until the end of fermentation, giving the final average counts of 9.8 and 8.9 log CFU/g for fermented BMM and BFM, respectively.

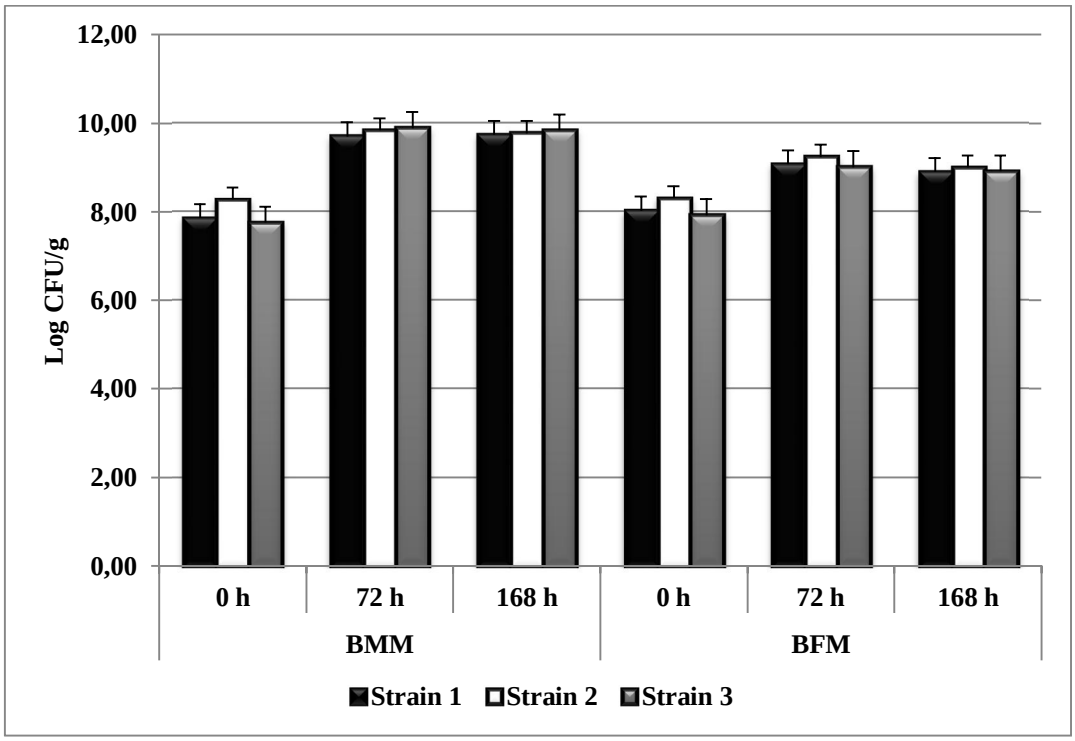

Figure 20. The cell counts (log CFU/g) of the *P. freudenreichii* strains 1, 2 and 3 in the cereal matrices during the 168 h-vitamin B_{12} production. BMM: 33% w/v barley malt and BFM: 6% w/v wholemeal barley flour.

3.3.3.2 Vitamin B_{12} Content

The vitamin B_{12} levels in the fermented cereal matrices were obtained from MBA since only few fermented cereal samples were analysed by the UHPLC method.

Barley Malt Matrix

Without the addition of any precursors, the fermented BMM contained 45.1, 23.1 and 21.5 ng/g of vitamin B_{12} (Figure 21), produced by the strains 1, 2 and 3, respectively. No increase of vitamin B_{12} contents was observed in any of the samples supplemented with DMBI. However, when DMBI and cobalt (II) chloride were added to the matrix, a considerable increase up to 40-fold was observed, yielding 1407.1, 1032.9 and 988.9 ng/g, produced by the strains 1, 2 and 3, respectively.

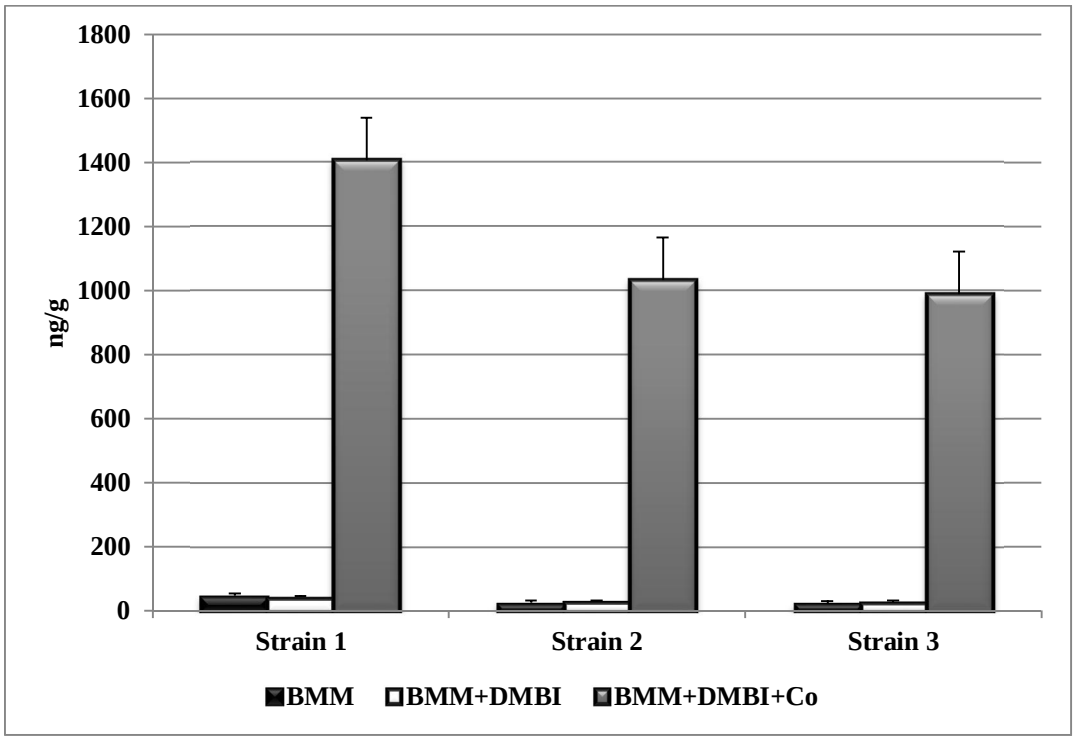

Figure 21. The vitamin B_{12} concentrations produced by the *P. freudenreichii* strains 1, 2 and 3 in the barley malt matrix, treated under three conditions during the 168-hour-vitamin B_{12} production (means and standard deviations of 3 biological replicates).

Barley Flour Matrix

The influence of DMBI and cobalt (II) chloride addition on vitamin B_{12} production in the fermented BFM showed similar trends (Figure 22) to the vitamin B_{12} production in the fermented BMM (Figure 21). However, all strains grown in the BFM produced relatively low levels of vitamin B_{12}; the maximum vitamin B_{12} production was achieved by the strain 1 at 80.1 ng/g.

When precursors were not added, strains 1, 2 and 3 produced 10.2, 8.0 and 12.2 ng/g of vitamin $B_{12,}$ respectively. No increase of vitamin B_{12} contents due to the addition of DMBI was observed. The combination of DMBI and cobalt (II) chloride increased the amount of vitamin B_{12} up to 8-fold, yielding 80.1 and 50.1 ng/g for the strains 1 and 2, respectively.

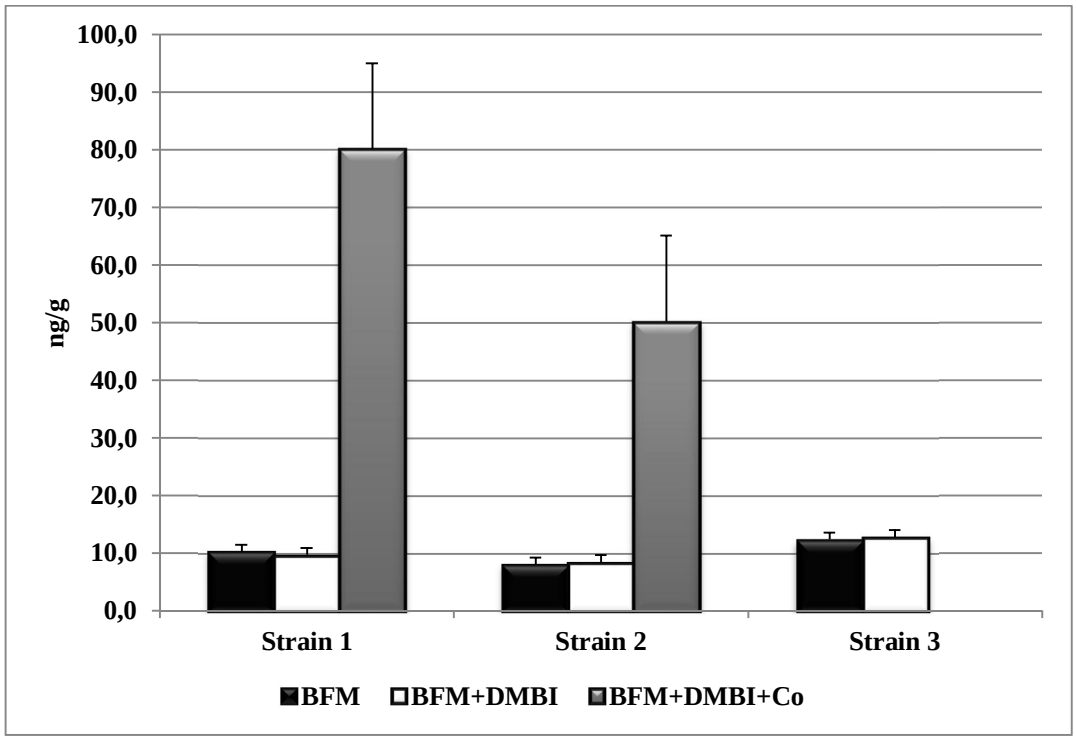

Figure 22. The vitamin B_{12} concentrations produced by the *P. freudenreichii* strains 1, 2 and 3 in the barley flour matrix, treated under three conditions during the 168-hour-vitamin B_{12} production (means and standard deviations of 3 biological replicates).

3.3.3.3 Analysis of Vitamin B_{12} by MBA and UHPLC

Vitamin B_{12} quantification by the UHPLC method was only applied to the fermented BMM samples due to the infeasibility to enrich the fermented BFM samples through immunoaffinity columns. Most of the samples analysed by the MBA showed higher vitamin B_{12} contents than those measured by the UHPLC method (Table 9), having discrepancy up to 113.2%. The amount of vitamin B_{12} in the samples influenced the variance between the two methods. In the samples supplemented with DMBI and cobalt (II) chloride, which contained quite much higher vitamin B_{12} had small difference of 4.7%, 9.0% and 4.8% for the strains 1, 2 and 3, respectively. While greater difference was observed in the samples that contained low levels of vitamin B_{12}.

Table 9. The vitamin B_{12} concentrations produced by the three *P. freudenreichii* strains in 33% w/v barley malt matrix, analysed by microbiological assay and UHPLC.

Conditions	Strains								
	1			2			3		
	Vit. B_{12} conc. (ng/g)			Vit. B_{12} conc. (ng/g)			Vit. B_{12} conc. (ng/g)		
	MBA	UHPLC	MBA/UHPLC	MBA	UHPLC	MBA/UHPLC	MBA	UHPLC	MBA/UHPLC
BMM	45.14±5.73[a]	37.14±3.20[b]	1.22	23.07±1.01[a]	10.96[c]	2.11	21.52±1.19[b]	10.09[c]	2.13
BMM+DMBI	40.68±3.18[a]	37.50[c]	1.08	27.30±2.60[a]	14.33[c]	1.68	26.16±1.53[a]	19.55[c]	1.34
BMM+DMBI+Co	1407.13±35.09[a]	1476.99±59.81[a]	0.95	1032.94±79.12[b]	1134.60[c]	0.91	988.95±11.33[a]	944.03±48.40[a]	1.05

[a] All values obtained represent mean ± SEM (n = 3)
[b] All values obtained represent mean ± SEM (n = 2)
[c] Values obtained from one replicate.

3.4 Discussions

3.4.1 Effect of Precursors on the Growth of *Propionibacterium* Strains

The pH of the SWP medium decreased from pH 6.5 to pH 5.0-5.3 after 72 h of fermentation and further declined to pH 4.9-5.0 at the end of fermentation. This pH profile was typically found in all fermentations. Utilizing *P. freudenreichii* and *Propionibacterium sp.*, the pH profile during vitamin B_{12} production in SWP medium done by Hugenschmidt et al. (2010) was alike; the pH of the cultures was between 5.5-6.2 after 72 hours and decreased to less than 5.0 after 168 hours of fermentation.

Depending on the three *P. freudenreichii* strains, slight differences of pH in the SWP medium were observed after 168 hours of fermentation. Samples inoculated with the strain 1 had the lowest pH at 4.87 followed by the strain 2 and strain 3 at pH 4.95 and 5.04, respectively. The difference of the pH could be influenced by the type and the concentration of acids produced by each strain during the fermentation. To confirm this observation, analysis of organic acids could be performed, which was not included in this study.

Furthermore, the addition of various precursors did not seem to influence the pH of the SWP medium and the cereal matrices. This finding was in agreement with the results of Marwaha et al. (1983), who studied the influence of the DMBI addition on lactose utilization in vitamin B_{12} production by three strains of *Propionibacterium*. Utilizing whey permeate as the fermentation medium Marwaha et al. (1983) reported that the degree of lactose assimilation during vitamin B_{12} production was not influenced by the addition of DMBI at various time intervals (Figure 23a). Although lactose utilization was not measured in this study, besides producing vitamin B_{12}, the *P. freudenreichii* strains also converted lactose to organic acids such as propionic acid and acetic acid (Colomban 1993; Hunik 1999), which reduced the pH during the fermentation.

In the fermented BFM, the pH of the samples remained close to the initial pH of the matrix (Figure 19), which could be influenced by the lower availability of carbon sources in the BFM (6% w/v) than in the BMM (33% w/v). Since the BFM contained fewer sugars and other nutrients to be utilized by the *P. freudenreichii* strains, lower concentration of organic acid could have been produced, explaining

no change in the pH of at the end of fermentation. Organic acids analysis could also be performed to confirm this observation.

Based on the OD in the SWP medium (Figure 14 and 17), the strain 1 showed approximately twice higher OD than the OD of the other two strains. The availability of carbon sources in the cereal matrices might also influence the cell counts. The strains grown in the BMM had approximately a log factor higher cell counts than the strains grown in the BFM (Figure 20). Both the OD and cell counts revealed that the addition of cobalt, DMBI, riboflavin and and nicotinamide neither enhance nor inhibit the growth of *P. freudenreichii* strains. Interestingly, the addition of riboflavin/nicotinamide at different phases of fermentation enhanced the growth of *P. freudenreichii* strains, particularly the strain 3 (Figure 17). The addition of riboflavin/nicotinamide at 0 and 72 h markedly increased the OD of the strain 3. While a slight increase of OD was observed when the precursors were added at 144 h. The effect of timing of adding precursor on the growth of the cultures during vitamin B_{12} production was also reported by Marwaha et al. (1983). Although they did not test the effect of riboflavin/nicotinamide, they observed the influence of DMBI addition at different phases of fermentation on the growth of *Propionibacterium* cultures (Figure 23b). Contrary with the addition of riboflavin/nicotinamide, the study showed the maximum biomass concentrations were achieved when DMBI was added after 144 h of fermentation and the addition of DMBI at early stages of fermentation inhibited the growth of the organisms.

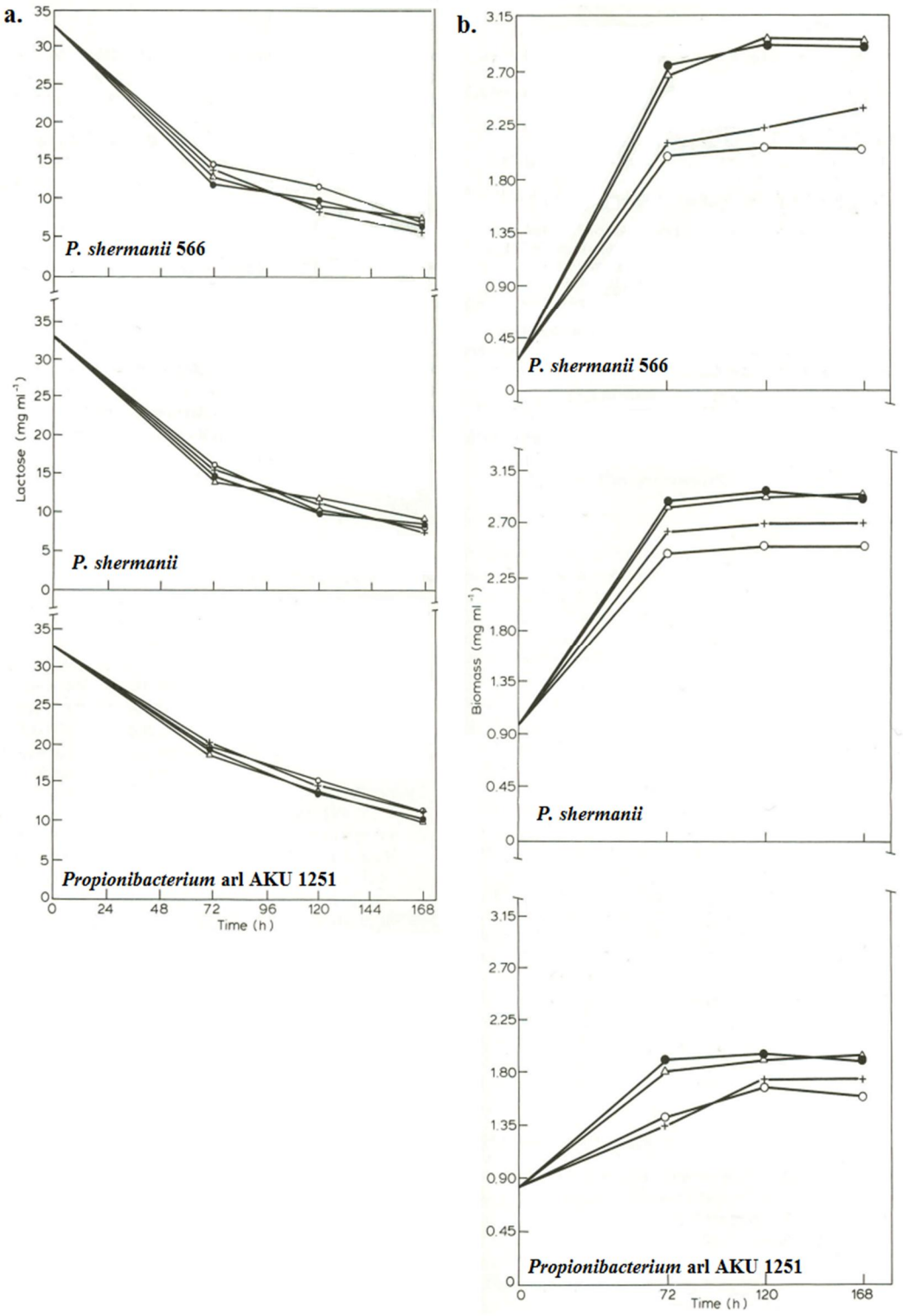

Figure 23. (**a.**) Influence of DMBI addition on lactose utilization during vitamin B_{12} production; (**b.**) The effect of timing of DMBI addition on the growth of three *Propionibacterium* cultures: *P. shermanii* 566, *P. shermanii* and *Propionibacterium* arl AKU 1251. ○, 0 h; +, 48 h; ●, 96 h; Δ 144 h (Marwaha et al. 1983).

3.4.2 Effect of Precursors on Vitamin B_{12} production

3.4.2.1 Effect of Cobalt Supplementation

The availability of cobalt is crucial for the synthesis of vitamin B_{12}. As the central atom of the planar ring system in vitamin B_{12} structure, cobalt has been suggested as the limiting factor for vitamin B_{12} biosynthesis (Panzeca et al. 2008). 5 mg/L cobalt (II) chloride was added in this study, as suggested by Hugenschmidt et al. (2011), who obtained a maximum vitamin B_{12} yields after testing different cobalt chloride concentrations (0, 0.01, 0.05 and 5 mg/L) in SWP medium without the DMBI addition. The addition of cobalt (II) chloride largely affected the vitamin B_{12} yields of *P. freudenreichii* strains in this study. Without the addition of cobalt or any other precursors, all three strains produced the lowest but also similar vitamin B_{12} yields, either in the SWP medium or the cereal matrices. An increase of vitamin B_{12} yields up to 4-fold was observed in the cobalt supplemented-SWP medium depending on the strains (Figure 18).

3.4.2.2 Effect of DMBI Supplementation

In addition to cobalt, the availability of DMBI (the lower ligand of vitamin B_{12}) is also crucial in forming the vitamin. When DMBI is absence or replaced by other substituents, such as adenine, analogues of vitamin B_{12} called pseudo-vitamin B_{12} are formed (Martens et al. 2002). These corrinoids have no vitamin activity in mammals (Gregory, 1996).

Despite the ability of *P. freudenreichii* strains to grow under aerobic conditions, the maximum production of corrinoids (precursors of vitamin B_{12}) is achieved under anaerobic fermentation (Quesada-Chanto et al. 1998). On the contrary, the biosynthesis and attachment of DMBI in *P. freudenreichii* strains require oxygen (Renz 1970). Thus, to achieve maximum vitamin B_{12} yields in our study, the vitamin B_{12} production by *P. freudenreichii* strains was performed in two-stage fermentation consisting of 72 hours of anaerobic incubation followed by 96 hours of aerobic fermentation, as suggested by Martens et al. (2002).

Depending on the strains, it has been shown that propionic acid bacteria are able to produce their own DMBI (Friedman and Cagen 1970), but it is often added to increase vitamin B_{12} yields. In this study, DMBI was added at the later stage of fermentation (144 h) since supplementation of DMBI at early stages of

fermentation inhibits the growth of *Propionibacterium*, resulting lower yields of vitamin B_{12}. The low yields of B_{12} with early addition of DMBI could be due to the increased energy consumption by the cultures to overcome the inhibitory effect of DMBI rather than its utilization for the growth and metabolite formation processes (Marwaha et al. 1983).

The two-stage fermentation and the addition of 15 mg/L DMBI in cobalt-supplemented SWP medium led to maximum vitamin B_{12} production by the strain 1 and 2, yielding 1.7 and 1.9 µg/mL, respectively. These results were comparable with the study conducted by Hugenschmidt et al. (2010), who achieved maximum vitamin B_{12} production at 2.5 µg/mL, produced by *P. freudenreichii* DF15 in SWP medium supplemented with 5 mg/L cobalt (II) chloride and 15 mg/L DMBI. Without the DMBI addition, the strain 1 and 2 produced approximately one third of vitamin B_{12} yields in the cobalt-supplemented SWP medium. On the contrary, compared to the vitamin B_{12} yields in the cobalt-supplemented SWP medium (Figure 15 and 18), insignificant increase ($P=0.065$) of vitamin B_{12} yields due to DMBI addition was observed in the samples fermented with the strain 3. When cobalt was not added, low vitamin B_{12} yields were observed in DMBI-supplemented cereal matrices with the amount below 50 ng/g of vitamin B_{12}. However, the combination of cobalt and DMBI markedly increased the vitamin B_{12} yields up to 8- and 40-fold in the BFM (Figure 22) and the BMM (Figure 21), respectively.

3.4.2.3 Effect of Riboflavin/Nicotinamide Supplementation

P. freudenreichii was demonstrated to utilize riboflavin as precursor in DMBI biosynthesis (Renz 1970) whose transformation appeared to be stimulated by nicotinamide (Hoerig and Renz 1980). Therefore, this study investigated the effect of riboflavin/nicotinamide addition on vitamin B_{12} production. According to the level of vitamin B_{12} produced in this study, the capacity of *P. freudenreichii* strains in vitamin B_{12} production varied depending on the type of precursors added in the SWP medium. As shown in Figure 15, the strain 1 and 2 reached maximum vitamin B_{12} production when cobalt-added SWP medium was supplemented with DMBI. While under the same conditions, the strain 3 produced maximum vitamin B_{12} yields when riboflavin/nicotinamide was added.

These observations were in agreement with the results of Hoerig and Renz (1980), who studied the DMBI-forming system in two subspecies of *P. freudenreichii* strains: *P. freudenreichii subsp. freudenreichii* and *P. freudenreichii subsp. shermanii*. They found that intact cobalt-free-grown *P. freudenreichii subsp. freudenreichii* ATCC 8207 cells produced DMBI from riboflavin whereas under the same conditions *P. freudenreichii subsp. shermanii* St 33 cells formed only trace amounts of DMBI, confirming *P. freudenreichii* strains react differently towards the addition of DMBI, riboflavin and nicotinamide. This observation thus showed the diversity of *Propionibacteria* in terms of DMBI-forming system on vitamin B_{12} production. Despite acknowledging that the three *P. freudenreichii* had different capacity of vitamin B_{12} production in the presence of riboflavin/nicotinamide, the exact mechanism of how these three strains utilized riboflavin/nicotinamide to form DMBI was not well-understood.

3.4.3 Effect of Timing of Riboflavin/Nicotinamide Supplementation

The timing in adding riboflavin/nicotinamide largely affected the vitamin B_{12} concentrations in the SWP medium. Both strain 1 and 3 synthesized higher vitamin B_{12} yields when riboflavin/nicotinamide was added at the beginning of fermentation than at the latter stage. However, a significant increase of B_{12} yields was particularly observed in the samples inoculated with the strain 3. Compared to the vitamin B_{12} yields when riboflavin/nicotinamide was added at 144 h, the strain 3 produced 2-fold higher vitamin B_{12} concentrations when the precursors were added at 0 and 72 h (Figure 18), yielding 2.2 and 2.1 µg/mL of vitamin B_{12}, respectively. The effect of timing of riboflavin/nicotinamide addition on vitamin B_{12} yields could be due to the growth enhancement of *P. freudenreichii* strain 3 that was also observed with early addition of riboflavin/nicotinamide; allowing the strain to produce more vitamin B_{12}. Furthermore, since lag phase occurs when nicotinamide stimulates DMBI formation from riboflavin (Hoerig and Renz 1980), the addition of riboflavin/nicotinamide at early stages compensated the time consumed during the lag phase and gave longer time for the transformation of DMBI, providing more available DMBI. However, to confirm if more DMBI is formed when riboflavin/nicotinamide is added at early stages, the concentrations of DMBI should also be measured, which was not covered in this study.

3.4.4 Vitamin B_{12} Production in Cereal Matrices

Quantified by MBA (*L. delbrueckii* ATCC 7830), the maximum vitamin B_{12} yield in the cereal matrices was 1.41 µg/g, produced by the strain 1 in DMBI- and cobalt-supplemented BMM. This amount of vitamin B_{12} was markedly higher compared to the reported vitamin B_{12} yields in a commercial fermented cereal product, tempe. Tempe is made by soaking, dehulling and cooking soybeans (or other legumes, seeds and cereal grains). The soybeans are then surface-dried and inoculated with the spores of *Rhizopus spp.* (*R. oligosporus*, *R. stolonifer*, *R. arrhizus* and *R. oryzae*), forming compact patties during the solid substrate fermentation (SSF). However, the moulds do not synthesize physiologically active vitamin B_{12}. Instead, vitamin B_{12} is produced by contaminant bacteria such as *Bacillus megaterium*, *Cit. freundii*, *Enterobacter cloacae*, *Escherichia coli*, *Kl. pneumoniae*, *Kl. terrigena*, *Kl. planticola*, *Kl. ozeanae* and *Streptomyces olivaceus* that occur during soaking and/or SSF.

Liem et al. (1977) isolated and purified vitamin B_{12}-producing bacterium (unidentified) from tempe (Tjing Giok Tan, Toronto, Canada). Reinoculation of this bacterium into sterile soybean produced 0.15 µg/g of vitamin B_{12} (MBA; *L. leichmannii* ATCC 7830). Keuth and Bisping (1993) also investigated the vitamin B_{12} production in tempe and found *Cit. freundii* and *K. pneumoniae* were the best vitamin B_{12}-producing bacteria in tempe. By adding *Cit. freundii* (isolate 259) and *K. pneumoniae* (isolate 274) at the beginning of tempe fermentation, Keuth and Bisping (1994) achieved 0.15 and 0.14 µg/g of vitamin B_{12} (MBA; *L. leichmannii* ATCC 7830) after 34 h of SSF at 32°C. With the high vitamin B_{12} produced in the BMM, this study hence showed the promising possibilities to produce high amount of vitamin B_{12} in other plant-based food matrices through *in situ* fermentation. The lower availability of sugars in the BFM (6% w/v) than in the BMM (33% w/v) might also influence the level of vitamin B_{12} in the fermented cereal matrices since relatively low levels of vitamin B_{12} produced in the fermented BFM (max. production of 0.08 µg/g). The lower concentration of substrate available in the BFM thus hindered *P. freudenreichii* strains to grow and achieve high production vitamin B_{12}. Furthermore, the grains in the barley malt flour were germinated via malting process. During germination, α- and β-amylases convert the starch into simple sugars such as glucose (Delcour and Hoseney 2010), which was readily

utilized by the *P. freudenreichii* strains, explaining much higher vitamin B_{12} concentrations produced in the fermented BMM.

In the cobalt- and DMBI-supplemented SWP medium inoculated with the strain 3 contained much lower vitamin B_{12} yields compared to the culture samples fermented by strain 1 and strain 2 (Figure 15 and 18). However, in cobalt- and DMBI-added fermented BMM, strain 3 produced approximately equal level of vitamin B_{12} yields to those produced by strain 2 (Figure 21). This may be influenced by the availability of riboflavin and niacin (vitamin B_3/nicotinic acid) in the cereal grains. Hoerig and Renz (1980) found that although nicotinamide stimulated the transformation of DMBI from riboflavin, nicotinic acid seemed to be the true stimulating agent as no lag phase was observed when nicotinamide was replaced by nicotinic acid. Moreover, the lag phase was also omitted when *P. freudenreichii* was pre-incubated with nicotinamide that was completely split to nicotinic acid after 3 h of incubation (Hoerig and Renz 1980). Barley malt flour naturally contain riboflavin (0.22 mg/100 g) and nicotinic acid (6.4 mg/100 g) (Delcour and Hoseney 2010), the two precursors that are involved in DMBI formation by *P. freudenreichii*. Since the strain 3 was observed to produce maximum vitamin B_{12} yields with the addition of riboflavin/nicotinamide, the availability of riboflavin and nicotinic acid in barley malt flour may increase the vitamin B_{12} yields in cobalt- and DMBI- supplemented BMM.

3.4.5 Vitamin B_{12} Quantification by Newly Developed UHPLC Method

Microbiological assay (MBA) has been the official reference method in vitamin B_{12} determination because of its high sensitivity and low-cost. However, MBA lacks of specificity as the indicator organism (*L. delbrueckii* ATCC 7830) may also react to vitamin B_{12} analogues and other corrinoids that are not active in humans, giving discrepancy approximately 5-30% compared to chromatographic methods (Ball 2005; Blake 2007). The HPLC/UV detection coupled with immunoaffinity extraction has been recently adopted for consideration as the future *Official Method* (AOAC Official First Action status) to determine vitamin B_{12} in selected food products (Campos-Giménez et al. 2008). However, the recently demonstrated UHPLC method offers quantification of vitamin B_{12} with higher chromatographic resolution and much shorter run time (Owen et al. 2011). Determined by the newly developed UHPLC method (Chamlagain et al. submitted), the maximum vitamin

B_{12} concentration in this study was 2.20 µg/mL, produced by the *P. freudenreichii* strain 3 in cobalt- and riboflavin/nicotinamide-supplemented SWP medium. This value was close to the maximum vitamin B_{12} production (2.5 µg/mL) achieved in the study done by Hugenschmidt et al. (2010). Almost all the samples analysed by MBA showed higher vitamin B_{12} contents than those measured by UHPLC method. Quantified by the MBA, high discrepancy up to 110.6% was observed in the fermented cereal samples, particularly in the samples that contained relatively low levels of vitamin B_{12} (Table 9). Since LC/UV usually lacks of sensitivity in detecting low quantities of vitamin B_{12} in food matrices (Viñas et al. 2003), the amount of vitamin B_{12} in the fermented cereal samples may influence the variance between the two methods. The observation was in accordance to a study done by Guggisberg et al. (2012), who reported a relative high factor between MBA- and HPLC-determined vitamin B_{12} yields in different meat products. Nevertheless, with sufficient amount of vitamin B_{12} concentrations in the samples, the newly developed UHPLC method is able to quantify low levels of vitamin B_{12} in food matrices.

4 CONCLUSIONS

Utilizing an optimized process of 72 hours of anaerobic followed by 96 hours of aerobic fermentation without controlling the pH, this study achieved high production of vitamin B_{12} by *P. freudenreichii* strains, with maximum production of 2.20 µg/mL and 1.41 µg/g produced in supplemented whey permeate (SWP) medium and cereal matrices, respectively. However, since *Propionibacterium* cannot grow below pH 5.0, higher vitamin B_{12} production can be achieved with pH control at approximately 7.0 in order to maintain the growth of the organisms (Martens et al. 2002). Furthermore, with the amount of vitamin B_{12} produced in the fermented cereal matrices, this study successfully demonstrated the possibilities to enrich plant-based foods with vitamin B_{12} through *in situ* fermentation.

This study tested four different precursors that have been previously used in vitamin B_{12} biosynthesis: 5 mg/L cobalt (II) chloride, 15 mg/L DMBI, 15.05 mg/L riboflavin and 3.29 g/L nicotinamide. Cobalt and DMBI are the two important parts that build the vitamin B_{12} structure. Thus, their presence was crucial for high vitamin B_{12} production, shown by approximately 8-fold and 40-fold increase of vitamin B_{12} yields in the fermented SWP medium and the cereal matrices, respectively, due to cobalt and DMBI supplementation. However, depending on the *P. freudenreichii* strains, the addition of DMBI did not always lead to maximum vitamin B_{12} production, which was observed in the culture samples inoculated with the strain 3. The strain 3 produced maximum vitamin B_{12} yields with the addition of riboflavin/nicotinamide, the two precursors that are believed to be involved in DMBI biosynthesis. With the availability of riboflavin and nicotinic acid in cereal grains and the ability of the strain 3 to effectively transform riboflavin to DMBI, the strain 3 was the best *P. freudenreichii* strain to naturally produce vitamin B_{12} in plant-based food system.

This study also showed that the optimum vitamin B_{12} production by the *P. freudenreichii* strain 3 with riboflavin/nicotinamide addition was significantly affected by the timing of adding the two precursors. Tested in the SWP medium with three different time points of riboflavin/nicotinamide supplementation (0, 72 and 144 h), the optimum vitamin B_{12} production by the strain 3 was achieved when riboflavin/nicotinamide were added at the beginning of fermentation (0 h). The addition of riboflavin/nicotinamide at early stages could provide more time for the transformation of DMBI from riboflavin, providing more DMBI for vitamin B_{12}

synthesis. Hence, higher concentration of vitamin B_{12} was produced. This study reflected the diversity of *P. freudenreichii* strains in producing vitamin B_{12} in the presence of different precursors. However, the exact mechanism of how these three strains utilized the precursors, in particular riboflavin and nicotinamide, to form DMBI was not well-understood. Therefore, it is prospective to further investigate the mechanism of *P. freudenreichii* strains in utilizing riboflavin and nicotiniamide in vitamin B_{12} biosynthesis.

With a flow rate of 0.320 mL/min and 10-minute duration of each run, the quantification of vitamin B_{12} by the newly developed UHPLC method proved to be simple and fast. Compared to the standard microbiological assay, the vitamin B_{12} concentrations in the fermented SWP medium measured by the UHPLC method presented satisfying values with expected discrepancy of 2-34%. However, higher discrepancy between the two methods was observed in the fermented cereal matrices, particularly in the samples that contained low levels of vitamin B_{12}. This observation was in accordance to previous review about LC/UV methods that usually lack of sensitivity and specificity in detecting low quantities of vitamin B_{12} in food matrices. The newly developed UHPLC method was proven as a promising alternative method to quantify vitamin B_{12} in fermented plant-based food matrices.

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